

A NOTE ON THE LYMPHATICS OF THE MIDDLE AND LOWER RECTUM AND ANUS

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TWO FIGURES

While the lymphatic vessels draining the anus and rectum have been studied and described from time to time, mostly by French and German anatomists, there are a few controversial points remaining. From the point of view of the clinician some of these points have become increasingly important in recent years. This is partly because of the role of the lymphatics in the dissemination of carcinoma of the rectum, and partly because of the present trends in surgery toward more frequent preservation of the sphincter mechanism.

For these reasons it was felt that additional work leading either toward a confirmation of the existing descriptions or toward some additional information would be desirable.

MATERIAL AND METHOD

Fifteen stillborn infants, or infants dying soon after delivery, were used. The lymphatics in the anorectal region were injected with colored materials, after which the fresh specimens were fixed in formalin solution for 48 hours. Dissections were then carefully made, using the binocular microscope when necessary. This procedure is essentially the classical one for demonstrating lymphatic vessels.

If insoluble colored particulate matter such as carmine, or finely divided carbon is placed in or beneath the skin, sooner or later it can be found in the regional lymphatic vessels and in the nodes to which these vessels run. While little is

definitely known as to how the particles get inside the lymphatics, it is probable that the widely distributed and extremely delicate capillary network is ruptured at the time the injections are made. In the living animal additional factors are involved. Clark and Clark ('18) watched the phagocytosis of carbon and carmine particles injected into the tails of tadpoles, while Kampmeier ('25) reported that in amphibian larvae, red cells were engulfed by lymphatics.

The actual methods and materials for injection in the present work were selected only after a careful study of the subject and some preliminary experiments carried out on rats. Higgins' India ink was finally selected as the tracer substance. It was diluted with an equal volume of isotonic saline solution and was neutralized to a pH of 7.2 with N/10 hydrochloric acid. The resulting mixture was found to be unstable and it was necessary to use it immediately after preparation.

Injections were made with a 2 cm³ syringe and a 27 gauge needle. The ink was injected both intradermally and subcutaneously in the perianal region under constant minimal pressure. No more than .2 cm³ was injected through any one puncture and the injection of that amount was carried out over a period of from 60 to 90 seconds. It was discovered that the skin lymphatics were more readily entered if the needles were rocked back and forth, thus breaking the walls of some of the channels. The ink usually diffused over a distance of 3 to 4 cm within 5 minutes.

Similar methods were used to demonstrate the lymphatics in the anal canal and rectum, the injections being made just under the mucosa of the rectal wall.

The above technique is essentially that used by Brockman and Krahl ('50) in a study of the lymphatics draining the rectum in the living dog.

OBSERVATIONS

Like most previous investigators, and particularly Poirier, Cuneo and Delamere ('04), Rouviere ('38), we were able to divide the lymphatic plexuses of the anus and rectum into in-

ferior, middle and superior regions. These regions are drained by efferent channels which differ in course and termination. The inferior region includes the perianal skin and the anal canal up to the mucocutaneous junction. The middle region begins above the mucocutaneous junction and extends upward above the attachment of the levator ani muscles. The superior

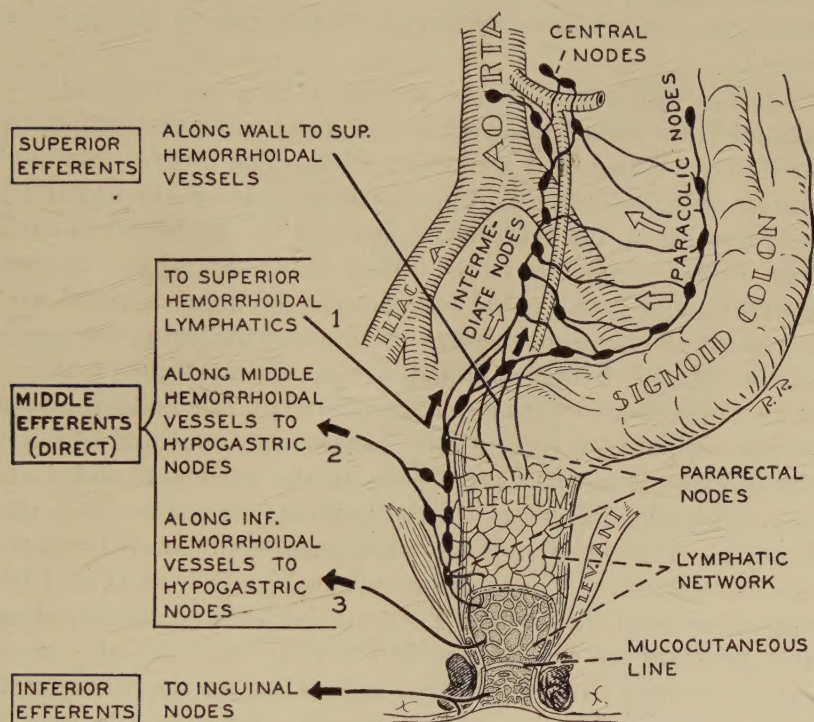


Fig. 1 The pattern of the lymphatic of the anus and rectum depicting the the inferior, middle and superior efferent channels.

region includes the upper part of the rectum. It is not sharply divided from the middle region which is also drained in part by the superior channels (fig. 1).

Inferior efferents. In the perianal region we were frequently able to fill two or three small lymphatic vessels on either side and follow them to the medial aspect of the thigh

and perineum, and thence to the superficial inguinal nodes. The lymphatics in the perianal region were found to be continuous with those of the anal canal but these channels apparently did not cross the mucocutaneous junction. All of our injections were limited sharply at that line (figs. 1 and 2). Injections into the wall of the lower anal canal gave similar results. The spread superiorly was again limited at the mucocutaneous line, while inferiorly spread was to the perianal region.

Middle efferents. In the middle region we were able to demonstrate both the direct and indirect efferent lymphatics of Poirier, Cuneo and Delamere ('04). The indirect channels were found ascending in the rectal columns of Morgagni and terminating in the lymphatic plexuses of the rectal mucosa. The direct channels were found to follow three general courses. One group could occasionally be demonstrated arising above the mucocutaneous junction and following the course of the inferior hemorrhoidal vessels. We were never able to trace these channels very clearly after they reached the lateral pelvic wall, but the indications are that they continue to follow the inferior hemorrhoidal vessels to the pudendal nodes and then the pudendal vessels to the hypogastric nodes (3 in figs. 1 and 2). These channels have not been emphasized by previous investigators. They were mentioned by Poirier et al. ('04) who considered them rare. These authors found similar channels perforating the levator ani muscle to reach the hypogastric nodes (? in fig. 2). Our injections, on the other hand, have never revealed any lymphatics perforating the levator ani muscles.

A second group of channels was more constant. In all cases we were able to follow 4 to 6 channels originating above the mucocutaneous junction and coursing laterally above the levator ani muscles. Two of these channels were quite consistently found to follow the course of the middle hemorrhoidal vessels. In a majority of cases, they were interrupted by nodes located at some distance from the lateral pelvic wall. In three specimens we were able to trace them to nodes surrounding the

hypogastric vessels (2 in figs. 1 and 2). These lymphatics have been described quite frequently (Quenu, Gerota, Mar-cille).

Superior efferents. The third group of channels from the middle region run parallel to the superior hemorrhoidal vessels and drain both the middle and superior regions (figs. 1

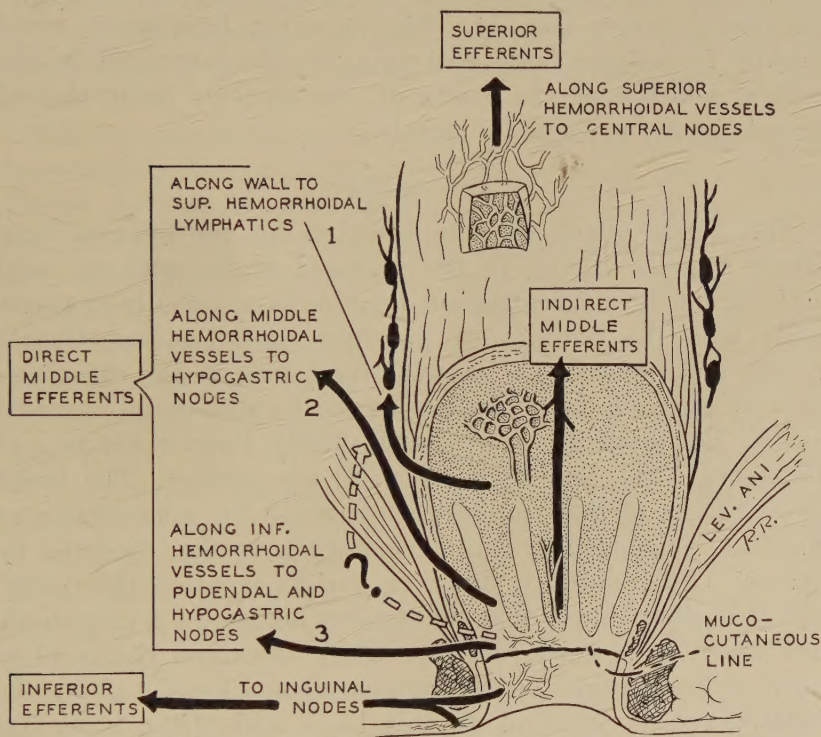


Fig. 2 A more detailed drawing of the lymphatic pattern of the lower and mid rectal regions. No lymphatics were found piercing the levator ani muscle.

and 2). In our experience, these channels were the most abundant and constant of all. They were adequately demonstrated in our injection studies. In attempting to fill these vessels, we started injections in the upper anal mucosa above the mucocutaneous junction and continued superiorly for about three inches, injecting .2 cm³ of ink every quarter of

an inch. We thus succeeded in filling one or two vessels draining superiorly in all instances. Collecting trunks from the superior region were found constantly to pass through the muscular coats at different levels and to continue superiorly and posteriorly to reach nodes in the mesorectum along the superior hemorrhoidal vessels. In one case we could demonstrate interruption of some of these channels in one of the pararectal nodes (fig. 1). The superior lymphatics could usually be demonstrated continuing to the intermediate and central nodes near the origin of the superior hemorrhoidal vessels at the brim of the pelvis.

DISCUSSION

The course which we demonstrated for the efferent lymphatic vessels arising in the perianal region and the anal canal below the mucocutaneous junction is essentially in agreement with the findings of previous investigators. Gerota (1895) described 2 to 5 vessels originating in the cutaneous part of the anus and terminating in the superficial inguinal nodes. The descriptions of Quenu (1893), Poirier, Cuneo and Delamere ('04), and Rouviere ('38) are similar. The break in continuity of the lymphatic plexuses at the mucocutaneous junction, suggested by our experiments, is also reported by Quenu. Other authors, however, fail to mention this point, and still others imply that connections across the mucocutaneous junction do exist. Mondor ('14) explained the involvement of the inguinal nodes from carcinoma located above the mucocutaneous junction on this basis and Miles ('25) has particularly emphasized the possibility of downward spread of cancer. It must be remembered, however, that anastomoses may occur between the efferent channels permitting retrograde spread when the superior channels are blocked. However, spread of carcinoma of the rectum to the inguinal nodes rarely occurs and then usually in late cases (Grinnell, '42 and Gilchrist and David, '38).

Of the efferent lymphatics draining from the middle region, the lowest group has not been mentioned frequently or estab-

lished as a constant route. Poirier et al. considered these lymphatics as being associated with the inferior hemorrhoidal vessels. They believed, however, that they rarely followed these vessels back to the internal pudendals and thence to the hypogastric nodes but reached the latter more directly by perforating the levator ani muscle (? in fig. 2). As stated previously, we have never been able to demonstrate any lymphatics perforating the levator ani but we were able to trace channels along the inferior hemorrhoidal vessels to the pudendal node area.

The second group of lymphatics from the middle region were considered as constant by Poirier et al. ('04). Quenu (1893) and Gerota (1895) had been able to demonstrate them only occasionally but according to Poirier, Marcille ('02) was able to find them in most cases. Rouviere also mentions these channels and describes additional lymphatics, which we were unable to trace, accompanying the lateral and middle sacral arteries. Villemin, Huard and Montagne ('25) mention lymphatic vessels following the nerves from the pelvic part of the rectum. We were able to constantly demonstrate the middle group of lymphatics draining the inferior rectal region. These channels are not, however, regarded as a constant route of early spread of carcinoma of the rectum (Grinnell, '42). Nevertheless, they are a potential course of spread for carcinoma of the rectum and particularly when the higher nodes are blocked.

The lymphatics generally considered as the most important are those running superiorly along the course of the superior hemorrhoidal vessels (1 in figs. 1 and 2). They have been noted by all modern investigators. Villemin, Huard and Montagne ('25) have described short, middle and long channels leading upward from the rectum. The majority of the channels which we have demonstrated belong to the short group. Rouviere ('38) speaks of these lymphatics as reaching nodes at the level of the bifurcation of the superior hemorrhoidal vessels. (The term he uses is inferior mesenteric, but Tobias in his translation of Rouviere's work points out that he is referring to

the superior hemorrhoidals.) We have clearly demonstrated that the superior lymphatic vessels drain part of the middle as well as the superior portion of the rectum (a conclusion shared by other investigators). This is in keeping with the tendency for spread of carcinoma of the rectum to nodes along the course of the superior hemorrhoidal vessels regardless of the location of the primary lesion (Grinnell, '42, Gilchrist and David, '38, Collier, Kay and MacIntyre, '40, and others).

SUMMARY AND CONCLUSIONS

In a series of 15 stillborn infants or infants dying soon after delivery, the lymphatics draining the anus and rectum were injected with a preparation of India ink and were studied grossly and under the binocular microscope.

The lymphatic plexuses of the anus and rectum were found to be divisible into inferior, middle and superior groups on the basis of the course and termination of the lymphatic channels draining them. The superior and middle region share some efferent channels although the middle region has additional channels of its own.

The inferior region, consisting of the anal canal and perianal skin, is drained by channels leading to the superficial inguinal nodes. We were unable to demonstrate continuity of the lymphatic plexuses of this area with those of the middle region above the mucocutaneous line.

The middle region, extending from the mucocutaneous line upward above the levator ani muscle is drained by efferent channels designated as indirect and direct. The indirect channels ascend in the rectal columns and terminate in lymphatic plexuses of the rectal mucosa higher up. There are three groups of direct channels perforating the rectal wall and proceeding in different directions.

(a) Below the insertion of the levator ani muscle, some lymphatics follow the course of the inferior hemorrhoidal vessels. We have not traced these channels beyond the lateral pelvic wall (pudendal area) and have never demonstrated any of them perforating the levator ani muscle.

(b) There are channels following the course of the middle hemorrhoidal vessels to reach the hypogastric nodes.

(c) A group of channels ascend the lateral wall of the rectum to continue with the efferents from the superior region. These vessels follow the course of the superior hemorrhoidal vessels to nodes in the sigmoid mesocolon and nodes above the brim of the pelvis along the inferior mesenteric vessels.

The superior region, or the higher portion of the rectum, is drained by the same channel as the third group from the middle region.

The results of this study suggest that although the main channels of spread for carcinoma of the lower and mid rectum are upward, there are definite channels for spread laterally along the inferior hemorrhoidal vessels on the inferior surface of the levator ani muscles and along the middle hemorrhoidal vessels on the superior surface of the levator ani muscles.

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CYTOCHEMICAL REACTIONS OF HUMAN SPERMATOOZOA AND SEMINAL PLASMA ¹

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NINE FIGURES

A number of recently developed histochemical methods makes it possible to localize and characterize various proteins, carbohydrates, lipids and enzymes within cells and tissues. The adaptation of some of these techniques to the staining of smears of blood cells on glass slides (Wislocki, Rheingold and Dempsey, '49; Bloom and Wislocki, '50) suggested that spermatozoa might be similarly prepared and studied.

MATERIAL AND METHODS

Specimens of human seminal fluid were secured from men between 20 and 26 years of age with no history of venereal disease. These were preserved in glass vials, the lower ends of which were submerged in an ice bath.² Smears of the specimens were prepared on glass slides within two hours of ejaculation. A sample of the fluid was examined under the microscope to establish that the spermatozoa were actively motile. A few smears of mouse spermatozoa obtained from the epididymis were used as a control for Baker's method for phospholipids. The smears were air dried for 1 to 24 hours

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² The material was obtained from the laboratory of Dr. Clarence J. Gamble.

before the slides were placed in the various fixatives. Preliminary drying proved essential in order to retain the smears on the slides.

The following fixatives and staining procedures³ were used:

Bodian's protargol method. After preliminary drying in air, the smears were fixed in Bodian's fixative no. 2 (80% ethyl alcohol 90 cm³, formaldehyde 5 cm³, glacial acetic acid 5 cm³). Staining was carried out according to Bodian's method as modified by Dawson and Barnett ('44). The smears were dehydrated, cleared and covered in clarite.

Sudan black B. After drying, the smears were fixed in 10% neutral formalin for 6 hours. They were then stained for 30 minutes to 1 hour in 70% alcohol saturated with sudan black B, dipped for a moment in 70% alcohol, washed in water and mounted in glycerine jelly.

Baker's acid hematein test for phospholipids. This procedure and the associated control (or pyridine extraction) test were carried out exactly as prescribed by Baker ('46). After preliminary drying, the smears for the hematein test were fixed in Baker's formalin-calcium chloride mixture for 6 hours and the control smears in Baker's "weak Bouin's fluid" overnight.

The periodic acid-Schiff procedure. After drying, the smears were fixed in Rossman's fixative (sat. sol. picric acid in abs. alc. 90 cm³, formaldehyde [added just before using] 10 cm³). The smears were then washed in distilled water, stained by the periodic acid-Schiff procedure according to the directions of McManus ('46), and then dehydrated, cleared and covered in clarite. Control smears were placed in saliva at room temperature for one-half hour before carrying out the periodic acid-Schiff procedure; in this way glycogen was removed. The preparations were counterstained with methylene blue, toluidin blue, hematoxylin or aniline blue.

³The assistance of Mrs. Edith Herman in the preparation of the material by the various techniques employed is thankfully acknowledged.

Pap's alkaline silver nitrate method. This method was applied to smears dried and fixed in Rossman's fixative. The procedure was carried out according to the modified technique of Mitchell and Wislocki ('44). Control smears were placed in saliva at room temperature for one-half hour before staining them.

Acid phosphatase. Smears were fixed in cold acetone for one hour at 4°C. after preliminary drying. Other smears were exposed to formalin vapor for three minutes at 44°C., according to a recommendation of Rabinovitch and Andreucci ('49) for smears of blood cells. After fixation, the acid phosphatase reaction was carried out at pH 4.7 for 12 and 24 hours according to the directions of Gomori ('41a), utilizing sodium glycerophosphate as well as fructose diphosphate and yeast-nucleic acid as substrates. The reaction turned out most successfully on smears that had been spread rather thinly upon the slides, for otherwise the very intense reaction of the seminal plasma almost completely masked the delicate spermatozoa.

Alkaline phosphatase. After preliminary drying, the smears were fixed in cold 80% alcohol at 4°C. overnight or in formalin vapor as outlined above. The reaction was carried out at pH 9.4 for 3, 6 and 12 hours by the method of Gomori ('41b) as modified by Dempsey and Deane ('46), utilizing sodium glycerophosphate and fructose diphosphate as substrates.

Staining with toluidin blue. The possible presence of metachromatic substances was investigated by staining smears in a $\frac{1}{2}$ % aqueous solution of toluidin blue for 30 minutes. The air dried smears were fixed overnight in a 4% aqueous solution of basic lead acetate before staining them. The smears were subsequently dehydrated, cleared and mounted in clarite. Metachromasia is generally accepted as being indicative of acid compounds, particularly acid mucopolysaccharides which may be conjugated with either sulfuric acid (mucoitin and chondroitin sulfates) or hyaluronic acid.

OBSERVATIONS

Bodian's protargol method. With this technique both the spermatozoa and the seminal plasma were delicately stained by the reduced protargol (fig. 1). In the spermatozoa the equatorial segment of the sperm head was clearly delimited by intense black staining of the borders of the anterior and posterior nuclear caps. The posterior border of the anterior nuclear cap showed up as a delicate line composed of minute black dots. The anterior border of the posterior cap, forming a second line bounding the equatorial segment, was also stained, being two or three times as wide as the previous line. The border of this second line presented an irregular, somewhat serrated appearance. In some of the spermatozoa the equatorial region between the two lines was almost colorless, but in others it was stained gray. Not infrequently blackish streaks extended completely across the equatorial segment. These variations are illustrated in figure 1. In most instances the anterior portion of the galea capitis was delicately stippled and there was some indistinct staining of the structures at the posterior border of the nucleus at the junction of spermhead and neck.

The precipitated seminal plasma exhibited well-defined black stippling (fig. 1).

Sudan black B. By this dye which stains lipids, the middle pieces of the spermatozoa were distinctly differentiated (fig. 2). The material which stained appeared frequently to be oriented in spiral fashion, corresponding to the so-called spiral filament, a structure generally believed to be of mitochondrial origin. Besides this distinct staining a faint gray tinge was usually perceptible in the equatorial zone of the head. The seminal plasma was not stained by this technique.

Baker's acid hematein test for phospholipids. The middle pieces of the spermatozoa were selectively stained by this procedure, although the spiral arrangement visible in the sudan preparations was not apparent (fig. 3). Preparations subjected to Baker's control procedure, consisting of extracting

the material with pyridine previous to staining it, showed no staining of the middle pieces (fig. 4). However, the nuclear substance in the sperm heads was stained, a feature characteristic of Baker's control test. Since Baker, himself, reported positive staining of the middle pieces of the spermatozoa in mouse testes, we stained smears of spermatozoa obtained from the epididymis of that animal in order to check our use of Baker's method; in these preparations the middle pieces were positive (fig. 5). The seminal plasma was negative following Baker's acid hematein test but was stained bluish black in the control preparations.

The periodic acid-Schiff procedure. The spermatozoa were negative following this method of staining, both before and after the use of saliva. The seminal plasma, on the other hand, stained intensely (fig. 6). Besides a red network of plasma, bright red particles were visible, some of them free, some adherent to spermatozoa, and others contained apparently in scattered epithelial cells present in the smears (fig. 6). None of this staining was abolished by preliminary exposure of the smears to saliva.

Pap's alkaline silver nitrate method. This method which is ordinarily used for the impregnation of reticulum also stains glycogen as well as some other carbohydrates after appropriate fixation (Mitchell and Wislocki, '44). The spermatozoa were not specifically stained by this procedure. The seminal plasma consisted of a tan-colored network with black dots scattered within it, besides many similar dots adherent to the poorly defined spermatozoa.

Acid phosphatase. Both the spermatozoa and the seminal plasma were stained after this procedure. The range and variability of staining after fixation in acetone is shown in figure 7. The reaction in the sperm heads occurred usually in two lines constituting the anterior and posterior borders of the equatorial segment, resembling the pattern of deposition of silver described after Bodian's method (fig. 1). Two such spermatozoa are illustrated in figure 7. A faint reaction was also apparent in the anterior portion of the galea capitis,

in this respect also showing a resemblance to the Bodian silver preparations. Some sperm heads were more deeply stained, involving more of the galea capitis and the equatorial zone; two such spermatozoa are also shown in figure 7.

The neck portions of the spermatozoa appeared to be somewhat stained, especially the anterior part containing the distal centrosome. A reaction for phosphatase outlined the middle and tail pieces of the spermatozoa, without providing any distinguishing marks between the two (fig. 7).

The enzymatic reaction in the coagulated seminal plasma was extremely intense as illustrated in figure 7.

Smears fixed in formalin vapor, as recommended by Rabinovitch and Andreucci ('49) for smears of blood cells, were generally poorly stained but occasionally gave a reaction involving the sperm heads in the manner shown in figure 8.

Of the three substrates used, fructose diphosphate gave stronger reactions than either sodium glycerophosphate or nucleic acid, the latter being the least intense.

Alkaline phosphatase. The cell contours were occasionally faintly outlined when fructose diphosphate was used as substrate, maximum staining occurring in the region of the junction of head and neck (fig. 9). With the two other substrates the spermatozoa were negative. In the seminal plasma a slight brownish reaction occurred.

Reaction with toluidin blue. This dye stained the nuclei of the spermatozoa blue, coloring the posterior part of the nucleus more deeply than the anterior portion. The other parts of the spermatozoa were unstained.

The seminal plasma revealed occasional areas of pink metachromasia in otherwise bluish stained plasma. These metachromatic areas were encountered most frequently in what appeared to be thickly spread parts of the smears.

DISCUSSION

In the present study the reactions of spermatozoa and plasma of human seminal fluid have been investigated by a number of histochemical methods. Some of the reactions are

present in either the spermatozoa or the plasma, whereas others occur in both.

The presence of lipids. Sudan black B stains different kinds of lipids whereas Baker's acid hematein method is believed to be specific for phospholipids. As Baker observed, the middle pieces of the mouse's spermatozoa are stained by his method in sections of testis. Besides confirming that observation, the present study shows that the middle pieces of human spermatozoa are positive. The middle pieces, it should be noted, are generally accepted by cytologists as consisting of an axial protoplasmic thread surrounded by a lipoidal spiral sheath which is derived from the mitochondria of the spermatids (cf. Metz, '32). The spiral arrangement of the material stained by sudan black B is in harmony with that concept.

Acid and alkaline phosphatases. Histochemical reactions for these enzymes occur in both seminal plasma and spermatozoa. It is quite possible, however, that the enzymes are adsorbed on the surface of the spermatozoa instead of being present inside of them. The fact that the faint alkaline phosphatase reaction merely outlines the spermatozoa (fig. 9) suggests that this enzyme is adherent to the cell surfaces. The reaction of the acid phosphatase is somewhat different. In this case the enzyme is concentrated in special localities of the spermatozoa, being particularly evident at the borders of the equatorial segment of the head and in the galea capitis (fig. 7). It is worthy of note that silver is deposited in a similar pattern by Bodian's method (fig. 1), although Pap's alkaline silver nitrate does not bring out these structures. It will be recalled that the reaction for acid phosphatase and staining by Bodian's method are both carried out at similar pH, whereas Pap's silver nitrate reaction occurs at extremely alkaline pH. It seems likely that acid phosphatase, as well as the silver of the Bodian reaction, becomes concentrated in the sites noted as the result of special conditions. The granular and stippled patterns which the reactions show suggest that the surface of the galea capitis and the borders of the equatorial segment possess a rough or uneven texture. This factor

would presumably favor the deposition and retention of substances on the surface of the spermatozoa.

In connection with the question of the manner of the localization of phosphatases in spermatozoa the observations of McCleod and Summerson ('46) are interesting. They found that human spermatozoa washed free of seminal fluid in vitro could not split the phosphate group at pH 7 from b-glycero-phosphate, glucose-1-phosphate, hexose-6-phosphate and fructose diphosphate, although they could hydrolyze adenosine triphosphate. The seminal plasma, on the other hand, could split phosphate from all of the enzymes used.

These observations show that at pH 7 the enzyme responsible for splitting all of the substrates used, with one exception, could be removed from the spermatozoa by thorough washing. Despite these observations carried out in vitro at pH 7, the cytological observations reported here indicate that, normally, acid phosphatase is so intimately associated with the spermatozoa that it would probably remain attached and be conveyed by them in their passage through the female reproductive tract. Thus, a possible means exists for transport by the spermatozoa of enzymatic constituents of the seminal plasma which would not otherwise reach the upper segments of the female reproductive tract. Moreover, the manner of localization of acid phosphatase suggests the possibility that the spermatozoa could adsorb similar substances which they might encounter during their passage through the uterine lumen and tubes.

The periodic acid-Schiff reaction. This procedure was introduced by McManus ('46) and interpreted chemically by Hotchkiss ('48) who concluded that polysaccharides are oxidized by periodic acid to give polyaldehydes which yield colored compounds with Schiff's leucofuchsin reagent. As Leblond ('50) has pointed out, the reaction is given by various substances some of which are readily soluble in water, some soluble in fat solvents and others soluble in neither water nor fat solvents. Under the conditions of fixation and staining

used in the present investigation, the method should reveal (1) glycogen, which may be removed from the specimens with saliva, (2) mucopolysaccharides (e.g., mucus [mucoitin sulfuric acid) and the ground substance of many connective tissues (hyaluronic acid), and (3) mucoproteins, (e.g., gonadotrophic hormones, seromucoid, etc.).

The present observations reveal that the seminal plasma is strongly periodic acid-Schiff positive but that the spermatozoa are negative (fig. 7). The substance responsible for the reaction of the plasma is not glycogen because it cannot be removed by saliva. Because of the intense reaction which it gives it is presumably a mucopolysaccharide, since known mucoproteins stain relatively faintly. Goldblatt ('35) from chemical analysis has reported the presence of traces of protein material, including mucin and albumin, in human seminal fluid. According to Hotchkiss, crude serum albumin gives a vigorous reaction by the periodic acid-Schiff test. Yet, the traces of mucin and albumin reported by Goldblatt would hardly seem adequate to account for the intense periodic acid-Schiff reaction observed in the seminal fluid smears. Moreover, the metachromasia, which it seems reasonable to ascribe to the presence of mucin, is faint and occasionally present only in the thicker parts of the smears. Thus the metachromatic reaction which should be attributed to acid mucopolysaccharides does not coincide with the uniformly distributed, intense periodic acid-Schiff reaction. Hence, the latter response should probably be ascribed to an unidentified mucopolysaccharide of neutral rather than acid character.

In spermatozoa in the testes of Virginia deer, the acroblasts and acrosomes of the spermatids, as well as the head caps of the spermatozoa, react positively by the periodic acid-Schiff method (saliva controls), a result indicating the presence of a mucopolysaccharide (Wislocki, '49). Similar observations have been reported in the spermatids and spermatozoa in rats' testes (Leblond, '50). Leblond, Clermont and Cimon ('50) reported further for rats as well as other species that, when the spermatozoa are fully mature and about

to be released from the Sertoli cells, the head caps lose their reactivity, so that in the epididymis and beyond, the head caps do not stain at all. They postulated from this that "the loss of polysaccharides is associated with a loss of viscosity which facilitates the release of the spermatozoa into the lumen of the seminiferous tubules."

The absence of Schiff reactive material in mature human spermatozoa accords with the observation of Leblond et al. that the spermatozoa of various species lose their staining as they mature. Nevertheless, the second generalization which relates the loss of viscosity to the loss of polysaccharides with release of the spermatozoa, seems less tenable, in view of the spermatozoa of the Virginia deer which, although they are in the ductus epididymis, still exhibit intense staining of their head caps (Wislocki, '49; figs. 16 and 29). Thus some other factor than the one suggested by Leblond would seem to be involved in the ultimate disappearance of the reaction.

Marza ('31) utilizing iodine methods (without reference to saliva controls) reported the presence of glycogen in the necks and middle pieces of spermatozoa obtained from the epididymis and seminal fluid of man, rat and various other animals. However, the periodic acid-Schiff technique with saliva controls has not revealed glycogen in the spermatozoa of Virginia deer (Wislocki, '49), rat (Leblond, '50) or man (present investigation). Since the periodic acid-Schiff method is a very sensitive one for glycogen (Lillie et al., '48), Marza's observations seem doubtful.

The several observations on spermatozoa of various animals stained by the periodic acid-Schiff technique suggest that the cytochemical properties of the sperm cells change during different phases of their development. These fragmentary data indicate the desirability of investigating spermatozoa further during their various stages by histochemical techniques.

SUMMARY

Various cytochemical methods have been adapted to the staining of smears of human seminal fluid. The galea capitis

of the spermatozoa and the borders of the anterior and posterior caps bounding the equatorial segment are intensely stained by Bodian's protargol method. These same structures as well as the distal portion of the neck react also for acid phosphatase. A reaction for alkaline phosphatase occasionally tinges the cell contours. The middle pieces stain black with Baker's acid hematein method for phospholipids and sudan black B. The spermatozoa of human seminal fluid are not stained by the periodic acid-Schiff procedure, indicating the absence of glycogen and other carbohydrates. The cells do not exhibit metachromasia following staining with toluidin blue.

The seminal plasma reacts strongly with the periodic acid-Schiff reagents but contains no glycogen (controlled with saliva tests). Occasional patches in the plasma stain metachromatically, a reaction attributed to the presence of small amounts of mucin. In view of this limited metachromatic reaction indicative of mucin, the intense and widespread periodic acid-Schiff reaction is attributed to the presence of a neutral mucopolysaccharide. The plasma reacts intensely for acid phosphatase but contains only traces of alkaline phosphatase. It exhibits a black stippling after Bodian's protargol method and stains brown with Pap's ammoniacal silver nitrate. It does not stain with either sudan black B or Baker's acid hematein test (in Baker's control test it is stained black).

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PLATES

PLATE 1

EXPLANATION OF FIGURES

All of the figures on this plate, excepting 5, are human spermatozoa as observed in smears of seminal fluid. For details of how the specimens were prepared consult the section on Methods. Drawn with an $\times 90$ objective and $\times 10$ ocular.

1 Spermatozoa and seminal plasma, stained by Bodian's protargol method. Fixation in Bodian's fixative no. 2.

2 Spermatozoa, stained with sudan black B. Fixation in 10% neutral formalin.

3 Spermatozoa, stained by Baker's acid hematein method. Fixation in Baker's formalin-calcium chloride.

4 Spermatozoa, stained by Baker's control method (pyridine-extraction). Fixation in Baker's "weak Bouin's fluid."

5 A spermatozan obtained from the epididymis of a mouse. Stained by Baker's acid hematein method.

1

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4

E. P. Ott

PLATE 2

EXPLANATION OF FIGURES

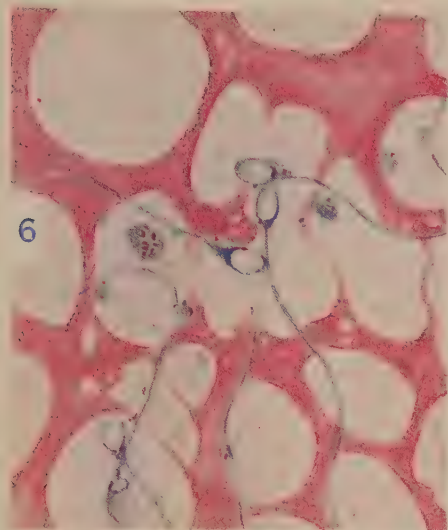
All of the figures on this plate are human spermatozoa as observed in smears of seminal fluid. For details of how the specimens were prepared consult the section on Methods. Drawn with an $\times 90$ objective and $\times 10$ ocular, excepting figure 6 for which a $\times 7$ ocular was used.

6 Smear of seminal fluid, stained by the periodic acid-Schiff procedure and counterstained with aniline blue. Treated with saliva before being stained. Fixation in Rossman's fluid.

7 Spermatozoa and a sample of seminal plasma, stained by Gomori's method for acid phosphatase. Smear incubated in sodium glycerophosphate for 48 hours at pH 4.7. Fixation in acetone.

8 Spermatozoa, stained by Gomori's method for acid phosphatase after fixation in formalin vapor. Incubated in fructose diphosphate for 24 hours at pH 4.7.

9 Spermatozoa, stained by Gomori's method for alkaline phosphatase. Smear incubated in fructose diphosphate for 12 hours at pH 9.4. Fixation in 80% ethyl alcohol.



E. Picotti

TRANSPLANTATION OF HEMATOPOIETIC TISSUES INTO THE CIRCULATING BLOOD

II. INJECTION OF BONE MARROW INTO NORMAL RABBITS, WITH SPECIAL REFERENCE TO THE HISTOGENESIS OF EXTRA- MEDULLARY FOCI OF HEMATOPOIESIS ¹

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TEN FIGURES

INTRODUCTION

In the previous report (Osogoe, '50), it was demonstrated that the cellular elements of lymph nodes, particularly the lymphocytes, when injected in large numbers into the circulating blood of normal rabbits, accumulate selectively in the periportal tissue spaces of the liver and the perifollicular areas of the spleen, during a period of 48 to 72 hours after the injection. In another series of experiments, a large amount of thymic tissue was injected intravenously into normal rabbits, and a selective accumulation of thymocytes took place in the periportal areas of the liver and the perifollicular regions of the spleen during the same period (Osogoe and Hitachi, '50).

The purpose of the present study is to observe the behavior of the bone marrow elements transplanted into the circulating blood and to use these observations, first, to compare them with those of the lymphoid cells in previous experiments, and second, to approach the study of the histo-

¹ This work was supported in part by a grant from the Department of Education for scientific research. A preliminary report covering certain phases of this study was read by one of the authors, B. O., at the 33rd meeting of the Japanese Pathological Society in Tokyo, April 1943 (Trans. Soc. Path. Jap., 33: 279-281).

genesis of centers of extramedullary hematopoiesis experimentally.

Two possible modes of establishment of extramedullary hematopoiesis are recognized: (1) local or "autochthonous" formation and (2) "colonization" of myeloid cells by metastasis from the bone marrow. Since myeloid transformation appears most commonly in organs which functioned as the chief sites of the blood formation in fetal life, particularly in the spleen and liver, the process is generally considered to be a return to the embryonal hematopoiesis. The majority of investigators, therefore, believe in an autochthonous formation of myeloid tissue through metaplasia from local fixed cells such as vascular endothelium, perivascular adventitial cells, or other connective tissue cells, and the process is often called "myeloid metaplasia" (e.g., by Naegeli, '31; Lang, '38; Rohr, '40).

The idea of "colonization" on the other hand, which was devised by Helly ('06), Ziegler ('06) and Askanazy ('11) has been abandoned to a large extent (Lang, '38).² Livadas ('33), however, demonstrated that myeloid transformation in the spleen and liver may occur as a result of colonization of the bone marrow cells within several hours after the injection of a large dose of sapotoxin.

MATERIAL AND METHODS

Normal rabbits over two months of age were used as recipients. Preliminary observations revealed that in such rabbits no hematopoietic foci occur in the spleen, liver or other organs, with the exception of the thymus in which a large number of myelocytes occur even in the adult.³ To avoid

² For a complete list of literature on this subject up to 1937, readers are referred to the review by Lang ('38). See also the monographs by Naegeli ('31) and Rohr ('40).

³ According to the unpublished observations by Ômura, myelocytes occur in the subcapsular layer of the thymus of the rabbit in large numbers. Their number increases as the thymus grows until its involution, particularly in the premature period.

a confusion of the results, therefore, data from the thymus are not included in the following description.

The marrows were taken out from the femur, tibia and humerus of two to 7 adult rabbits and made into a heavy suspension in a physiologic salt solution. Some difficulties were encountered in removing the fat globules without damaging the marrow cells. We succeeded in obtaining a sufficiently great number of uninjured cells, however, by grinding small cut pieces of marrow gently on a piece of double-folded gauze and washing out the cellular elements in saline. The fat globules were removed by repeated centrifuging. Suspensions containing as many as two billion nucleated bone marrow cells were obtained.

The differential cell counts of the marrow suspensions thus prepared gave an average myeloid-erythroid ratio of approximately one myelocyte to 6 erythroblasts.

The whole amount of the suspension, 20 to 40 cm³, was injected as a single dose into the ear vein of the recipient as slowly as possible. The total number of the nucleated cells injected into one animal amounted to from 2,000 to 12,360 $\times 10^6$.

In most instances, the usual staining by hematoxylin (Mayer's acid hemalum) and eosin after fixing in Zenker formol was sufficient for discrimination of the varying stages of maturation of the marrow cells in tissue sections, except for the earliest forms, myeloblasts and proerythroblasts, and basophilic myelocytes. The special hematoxylin and eosin-azur II method of Maximow was used only as an auxiliary. For discrimination of the earliest forms or other cells unidentified in sections, imprint preparations were made from the organs to be studied (lung, liver, spleen, etc.) and stained with Giemsa or May-Giemsa. The myelocytes in a broad sense were classified into three types, promyelocytes, myelocytes and metamyelocytes; and the erythroblasts into two types, macroblasts and normoblasts, both including basophilic, polychromatophilic and orthochromatophilic cells.

Differential counts of the bone marrow elements contained in 1 cm^2 of sections cut $8\text{ }\mu$ thick, or a volume of 0.8 cm^3 of organ, were made from different portions of the organs. According to Patten and Philpott ('21), after fixing in Zenker's fluid and imbedding in paraffin, the organs shrink to about 80% of their original volume. A volume of 0.8 mm^3 of fixed and imbedded tissues, therefore, corresponds closely to 1 mm^3 of the original fresh tissue so that data from the sections become comparable with those from blood counts. When the sections were cut $6\text{ }\mu$ thick, data obtained for 0.6 mm^3 were corrected to those for 0.8 mm^3 .

RESULTS

1. Distribution of the injected bone marrow elements in the blood and organs

The distribution of the injected marrow elements in the body of the recipient animals varied according to lapse of time and the different organs and tissues studied.

(a) *Peripheral blood.* The immature forms were found in the peripheral circulation only in small numbers (table 1), indeed, less than had been expected according to the enormous number of marrow cells introduced. The cells appearing in the peripheral blood were usually confined to normoblasts. A few metamyelocytes were occasionally seen (fig. 1). The number of the blood leucocytes was reduced during the first few hours, then markedly raised during the next 10 to 20 hours. These changes were due chiefly to decrease or increase in pseudoeosinophiles. In some instances, basophilic leucocytes appeared in relatively high numbers (more than 2%) in the period later than 24 hours after injection (table 1).

In the sections of the lung, liver and spleen, the marrow elements of both myeloid and erythroid series were found in much greater numbers than in the peripheral blood, as will be shown in the following.

(b) *Lung.* Immediately after the injection, as shown in table 2, the total number of myelocytes including pro- and

TABLE 1

Differential cell counts (500 cells) of the peripheral blood after injection of the bone marrow suspension. Rabbit no. 25, 2.4 kg ♂.
The total number of the injected nucleated cells was $2,600 \times 10^6$

	BEFORE INJECTION	1 HOUR	5 HOURS	18 HOURS	24 HOURS	48 HOURS	66 HOURS
Total number of white blood cells	7,385	4,136	6,508	17,211	8,320	6,044	5,500
Total number of nucleated red blood cells	15	264	92	209	..	36	..
Immature red cells (%)							
Proerythroblasts							
Macroblasts	0.2 ¹	6.0	1.4	1.2	..	0.6	..
Normoblasts							
Immature white cells (%)							
Myeloblasts		..	..	..	..	..	..
Promyelocytes		..	..	..	..	..	..
Myelocytes		..	..	..	..	..	..
Metamyelocytes		..	..	..	..	..	..
Mature white cells (%)							
Pseudoeosinophiles	41.0	22.6	50.4	48.4	46.6	47.6	26.6
Eosinophiles	1.4	0.2	..	2.2	0.4	..	0.6
Basophiles	..	0.4	1.2	2.4	1.8	2.4	9.0
Lymphocytes	45.2	58.0	44.0	38.2	43.0	39.0	50.2
Monocytes	12.0	12.8	3.0	7.6	8.2	10.4	13.6
Unidentified cells	0.2	..	..	..	..	..	..

¹A few nucleated red cells were occasionally seen in the peripheral blood of normal adult rabbits, although the animals lack any hematopoietic foci either in the spleen, liver or other organs.

metamyelocytes per 0.8 mm^3 of organ in the lung was 1,760, 10 times the 170 in the spleen or 16 in the kidney. None were found in the liver, lymph nodes and adrenals. The lung therefore is the initial organ in which the injected myelocytes are deposited. The erythroblasts did not have a similar preference for the lung, but a relatively high number of these cells did occur.

The marrow elements were found within the lumen of the capillaries of the alveolar walls and other small pulmonary

TABLE 2

Differential counts of the bone marrow elements per 1 cm^2 of section of the organs cut at 8μ (0.8 mm^3 of organ), 30 minutes after the injection. Rabbit no. 3, 1.3 kg ♂, the total number of the injected nucleated cells was $2,200 \times 10^6$

ORGAN	MYELOCYTES				ERYTHROBLASTS		MEGA-KARYO-CYTES
	Pro-M.	Eos. M.	Pse. M.	Meta-M.	Macro.	Normo.	
Lung	44	22	1386	308	88	484	21
Liver	..	..		...	399	1344	..
Spleen	..	..	170	...	408	1020	..
Lymph nodes ¹	..	..	...	...	..		..
Kidney	..	..	16	...	...	48	..
Adrenals	..	..	..	...	...	..	..

¹ The mesenteric lymph nodes.

Abbreviations: Pro-M., promyelocytes; Eos. M., eosinophilic myelocytes; Pse. M., pseudoeosinophilic myelocytes; Meta-M., metamyelocytes; Macro., macroblasts; Normo., normoblasts.

vessels, scattered rather diffusely over the whole organ, without any circumscribed cell collections as in the liver and spleen. Megakaryocytes, found in great numbers at the 24th hour, were for the most part of the naked nuclear and degenerating type (table 3). The marrow elements, especially those of larger size such as myelocytes and megakaryocytes blocked the small blood vessels of the lung as cell emboli soon after the injection. For this reason, few marrow cells appeared in the peripheral circulation.

The marrow elements in the lung were gradually decreased during the period from the 24th to 48th hour. At the same time, they accumulated to a great extent in the liver and spleen (table 3), but there was no significant increase in the number of marrow elements in the peripheral blood (table 1). It should be noticed that we missed typical myelocytes with round nuclei in all of the 5 examples carefully studied.

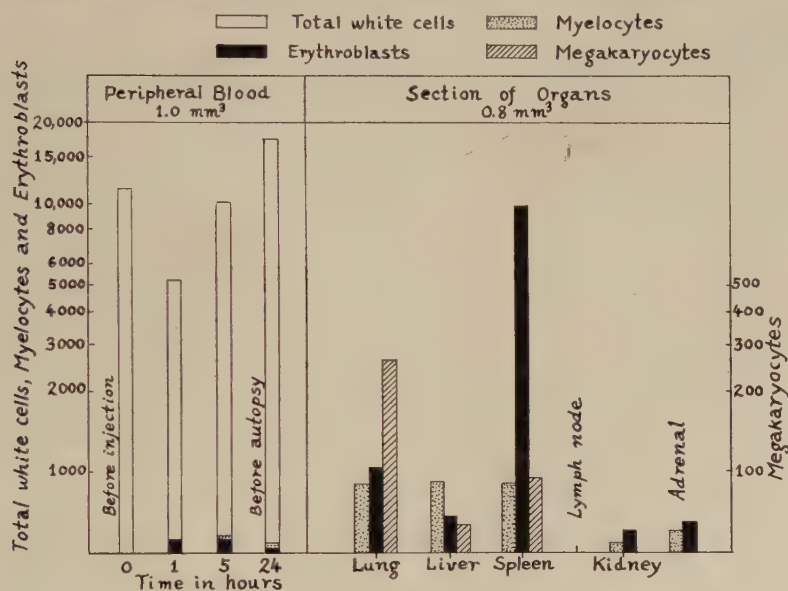


Fig. 1 Chart illustrating the number of the bone marrow elements contained per unit volume of the peripheral blood and sections of the organs (lung, liver, spleen, lymph nodes, kidney and adrenals) 24 hours after the injection. Rabbit no. 23, 2.25 kg ♀. The total number of the injected nucleated cells was $3,584 \times 10^6$. The graph is on logarithmic paper. The data of the right half of the graph is given in table 3.

(c) *Liver*. In the liver, bone marrow elements accumulated to a considerable degree during the period from the 48th to 72nd hour (table 3). Both myelocytes and erythroblasts were seen intravascularly, either scattered or grouped together as small cell collections, in the sinusoids between the liver cell cords (fig. 3). Megakaryocytes appeared singly in the lumen of the sinusoids. Topographically, the subcapsular layer was

TABLE 3

Differential counts of the bone marrow elements per 1 cm² of sections of the organs cut 8 μ thick (0.8 mm² of organ) during the period from the 24th to 72nd hour after injection

RABBIT NUMBER AND TIME AFTER INJECTION	TOTAL NUMBER OF INJECTED NUCLEATED CELLS	ORGAN	MYELOCYTES			ERYTHROBLASTS		MEGA- KARYO- CYTES
			Pro-M.	Eos. M.	Pse. M.	Meta-M.	Macro.	Normo.
No. 23, 2.25 kg ♀ 24 hours	3,584 $\times 10^6$	Lung	26	26	676 (52) ¹	78	234 (20) ¹	858
		Liver	84	..	560 (84) ¹	196	168	196
		Spleen	..	9	740	..	1,560 (18) ¹	8,090
		Lymph nodes ²	..	8	..	..	..	..
		Kidney	..	..	59	20	88	118
		Adrenals	22	22	143	11	77	209
No. 15, 900 gm ♂ 48 hours	2,300 $\times 10^6$	Lung	..	..	340	80	..	270
		Liver	50	10	1,330 (30) ¹	240	30	420
		Spleen	90	330	3,900 (120) ¹	540	480	32,640
		Lymph nodes ²	..	..	190 (10) ¹	..	..	50
		Kidney	..	..	180	..	..	30
		Adrenals	..	..	620 (20) ¹	20	..	890
No. 12, 1.9 kg ♂ 72 hours	8,720 $\times 10^6$	Lung	..	20	320	120	30	90
		Liver	60	10	1,310 (20) ¹	880	..	50
		Spleen	..	150	1,770 (60) ¹	660	660	2,100
		Lymph nodes ²	..	20	40	40	..	..
		Kidney	..	..	100	50	..	30
		Adrenals	..	..	10	..	..	20

¹ The number of mitoses involved.

² The mesenteric lymph nodes.

Abbreviations: Pro-M., promyelocytes; Eos. M., eosinophilic myelocytes; Pse. M., pseudoeosinophilic myelocytes; Meta-M., meta-myelocytes; Macro, macroblasts; Normo., normoblasts.

comparatively rich in marrow elements. In the differential cell counts of sections of the liver (table 3), the erythroblasts were always less numerous than myelocytes, which is remarkable because, in the injected marrow suspensions, the erythroblasts outnumbered the myelocytes 6 to 1. By contrast in the periportal spaces, myelocytes and erythroblasts were found exceptionally and in small numbers.

It is worthy of notice that in the animals which had received an extraordinarily great amount of marrow, there appeared in the periportal area large, diffuse and irregularly shaped collections of round cells which resembled erythroblasts, but could not be identified in the sections with certainty (fig. 4). The majority of such collections were not solely perivenous in position, but were in part within the interlobular portal vein. Within these collections typical erythroblasts (fig. 4) were often found.

In the liver imprints, the erythroblasts definitely predominated over the myelocytes, but the majority of the erythroblasts showed a variable degree of degenerative change which obscured the characteristic chromatin pattern by the 48th hour. It is logical, therefore, that in the sections such cells as accumulated in the periportal spaces could be identified as erythroblasts with difficulty and that the number of erythroblasts in the differential cell counts accordingly should be less than the myelocytes (table 3). Thus it is probable that the round cell collections of the periportal spaces are composed of the degenerating erythroblasts, with some typical erythroblasts intermingled, derived from the injected injured erythroblasts.

The myelocytes accumulated in the liver showed a large number of mitoses (table 3), and there was no indication of their rapid degeneration up to the 72nd hour. Besides myelocytes and erythroblasts, the earliest forms, myeloblasts and proerythroblasts, were also observed in the imprints of the liver but only in small numbers.⁴

⁴ The topographical distribution of myeloblasts and proerythroblasts was not clear, since a discrimination of these cells in the sections is hardly possible unless they appear in large numbers.

(d) *Spleen*. The spleen often became several times its normal size. The bone marrow elements accumulated in this organ more than in all the other organs during the period from the 48th to 72nd hour (table 3). The most interesting fact here is that the bone marrow elements as a whole accumulated in the red pulp, while the lymphoid cells (Osogoe, '50; Osogoe and Hitachi, '50) were almost exclusively in the perifollicular regions.

Myelocytes occurred for the most part in the meshes of the reticular stroma, particularly in the subcapsular layer of the red pulp, either scattered or grouped together in the form of diffuse cell collections (fig. 6). The erythroblasts, on the other hand, occurred in the venous sinuses where they were densely aggregated, often filling the dilated lumen almost completely (fig. 5). Megakaryocytes appeared singly not only in the venous sinuses but also in other parts of the red pulp. The marrow elements which had accumulated in the spleen, particularly the myelocytes, were in mitosis more frequently than those in the liver (table 3), and there was no indication of any degenerative changes of these elements up to the 72nd hour. In the imprints of the spleen, myeloblasts and proerythroblasts were more frequent than in the liver. In the spleen, in contrast with the liver, the erythroblasts always outnumbered the myelocytes. This tendency was particularly evident in the young animals (table 3).

(e) *Mesenteric lymph nodes, kidney and adrenals*. In these organs the majority of the bone marrow cells occurred intravascularly, either singly or grouped together as small clusters (table 3). In the mesenteric lymph nodes, they were found in the postcapillary veins (fig. 7); in the kidney, not only in the glomerular or other capillaries but also in the venules or larger veins; and in the adrenals in the sinusoids of the zona fasciculata and zona reticularis of the cortex (fig. 8). Mitotic figures of marrow elements were also seen in these organs (table 3).

In the kidney, small perivascular collections of marrow elements, particularly myelocytes, occurred along the relatively large veins where the successive stages of migration through the vascular wall could be traced. From 48 to 72 hours after the injection, small myeloid cell collections were often formed intravascularly on the wall of relatively large veins such as the arcuate vein and its cortical branches. At first, these collections adhered to the inner surface of the wall, like parietal thrombi, and were covered with fine fibrin threads (fig. 9). A proliferation of endothelial cells from the neighboring endothelium then spread along these fibrin threads, covering the free surface of these myeloid cell collections. Simultaneously, the original endothelium, no longer in direct contact with the blood stream, gradually underwent atrophy and disintegrated (fig. 2). As a consequence, the myeloid cell collections, formerly intravascular, became situated outside of the vessel.⁵ In some instances, the disintegration of the original endothelial barrier and subsequent emigration of myeloid cells into the perivascular connective tissue took place prior to the formation of new endothelium. In such cases the myeloid cells, particularly the myelocytes often infiltrated deeply into the perivascular connective tissue (figs. 2 and 10).

It is of particular interest that in the course of this process a new endothelial barrier often traversed the collections, dividing them into two parts, the extravascular composed mostly of myelocytes and the intravascular of erythroblasts. The myelocytes tended to migrate outside of the vessel, whereas the erythroblasts remained within its lumen. The same tendency, as already noted, manifests itself in the spleen where the myelocytes accumulate extravascularly in the meshes of the reticular stroma, while the erythroblasts collect intravascularly in the lumen of the venous sinuses of the red pulp.

⁵ The process is similar to the incorporation of the lymphocyte accumulations from the radicles of the interlobular portal vein into the periportal areas in the liver described in the first paper of the present series (Osogoe, '50).

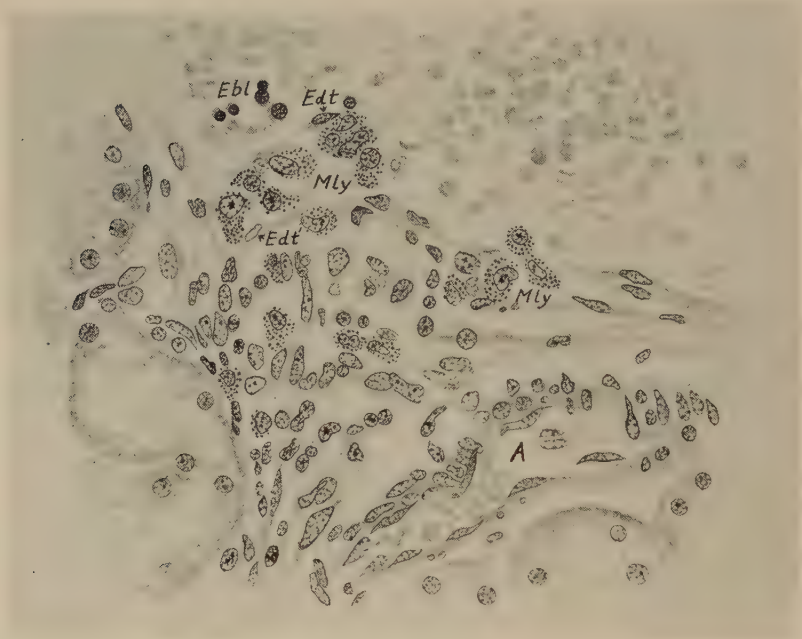


Fig. 2 Section through the kidney, showing two small myelocyte collections (Mly) on the wall of the arcuate vein. Notice the new formation of endothelium (Edt) covering the free surface of the left myelocyte collection, coincident with the disintegration of the original endothelium (Edt'). Several myelocytes are wandering deeply into the perivenous connective tissue having an arteriole (A). Also notice the erythroblasts (Ebl) remaining in the lumen. Forty-eight hours after the injection. Rabbit no. 15, 900 gm ♂. Camera lucida drawing from the preparation, fixed in Zenker formol and stained with Mayer's acid hemalum and eosin. $\times 800$.

2. Subsequent fate of the bone marrow elements accumulated in the liver, spleen and other organs

As seen in table 3 the bone marrow elements, especially the myelocytes, in the liver, spleen and other organs showed a number of mitoses up to the 72nd hour. Their subsequent behavior and fate was traced in a series of rabbits for two weeks.

After 72 hours the number of bone marrow elements in the liver, spleen and other organs decreased rapidly, so that by the 7th day they were in small numbers, confined to the spleen

TABLE 4
Control experiments; differential counts of the bone marrow elements per 1 cm² of sections of the lung, liver and spleen cut 8 μ thick (0.8 mm² of organ)

RABBIT NUMBER AND TIME AFTER INJECTION	TOTAL NUMBER OF INJECTED NUCLEATED CELLS	ORGAN	MYELOCYTES		ERYTHROBLASTS		MEGA- KARYO- CYTES
			Pro-M.	Eos. and Pse. M.	Meta-M.	Macro.	
Injection of the bone marrow elements killed by heating at 60°C. for 10 minutes							
No. 16, 1.95 kg 48 hours	12,360 × 10 ⁶	Lung	..	..	..	..	(264) ¹
		Liver	..	20	..	..	20
		Spleen	..	..	..	60	160
Injection of the bone marrow elements killed by freezing and thawing repeated twice							
No. 17, 1.7 kg 72 hours	7,081 × 10 ⁶	Lung	..	..	..	..	(16) ¹
		Liver	..	10	..	..	2
		Spleen	..	34	..	60	150
Injection of the lymph node elements killed by freezing and thawing repeated twice							
No. 35, 2.53 kg 48 hours	1,890 × 10 ⁶	Lung	..	..	..	..	..
		Liver	10	70	10	..	10
		Spleen	..	60	100	..	..

¹ Large, naked and shrunken nuclear remnants of megakaryocytes.

Abbreviations: Pro-M., promyelocytes; Eos. and Pse.M., eosinophilic and pseudoeosinophilic myelocytes; Meta-M., metamyelocytes; Macro., macroblasts; Normo., normoblasts.

and liver. The round cell accumulations of the periportal areas of the liver entirely disappeared on the 5th day. Two weeks after the injection, the marrow elements were no longer seen anywhere. The accumulated marrow elements had either differentiated into mature blood cells or undergone degeneration, and these cells were unable to continue to proliferate for a long time in these foreign situations.

3. *Control experiments*

In this group of experiments, the cells of bone marrow suspensions were killed by freezing and thawing twice or by heating at 60°C. for 10 minutes before injection. As shown in table 4, bone marrow elements were found in the liver and spleen but in much smaller numbers than in the first group of experiments and they were not in circumscribed cell foci. In other organs they did not appear at all. In some instances, myelocytes, erythroblasts and megakaryocytes occurred in the liver and spleen in somewhat greater numbers than had been expected. This is probably due to some extent to the effect of products of disintegration of the injected suspension, for, as noted in the previous paper (Osogoe, '50), the occurrence of these cells could also be induced by injections of killed lymph node elements (see the lowest division of table 4).

DISCUSSION

The sites of accumulation of bone marrow elements transplanted into the circulating blood are different from those of the lymphoid elements of the lymph nodes and thymus in earlier studies (Osogoe, '50; Osogoe and Hitachi, '50). In addition, between the myeloid and the erythroid cells, although both originate in the bone marrow, there is a difference in their sites of accumulation, particularly in the spleen. These differences in the behavior of cells of different strains is summarized in table 5.

Although some types of cells, especially the erythroblasts, are deposited in venous sinuses where the blood stagnates,

this cannot be fully explained by mechanical stagnation alone, since other types of cells accumulate extravascularly. We must consider, therefore, that the affinity of cells for organs and tissues differs according to differences in cell character. In connection with this, it is of particular interest that in leukemia the chief sites of accumulation or proliferation of leukemic cells differ according to the type. In lymphoid leukemia these sites are the periportal areas of the liver and the follicles of the spleen, whereas in myeloid leukemia the corre-

TABLE 5

Sites of the accumulation of different cell types in the liver and spleen after injection of suspensions of the hematopoietic organs

ORGAN	CELL TYPE	SITES OF ACCUMULATION	
		Liver	Spleen
Bone marrow	Myelocytes	Sinusoids	Meshes of reticular stroma of red pulp
	Erythroblasts	Sinusoids (Periportal spaces) ¹	Venous sinuses of red pulp
Lymph nodes	Lymphocytes	Periportal spaces	Perifollicular regions
Thymus	Thymocytes	Periportal spaces	Perifollicular regions

¹ The site of the accumulation of degenerating erythroid cells.

sponding sites are the sinusoids of the liver and the red pulp of the spleen (Richter, '38; Amano, '40).

Round cells resembling erythroblasts also accumulate in the periportal area of the liver as do lymphoid cells. This is probably due to a mechanical stagnation in the portal system which causes accumulation of the degenerating erythroblasts, or cells injured in the injected marrow suspension. It does not mean that the affinities of the erythroblasts are similar to those of the lymphoid cells, because the uninjured erythroblasts do not accumulate according to the pattern of lymphoid cells.

With regard to the histogenesis of extramedullary foci of hematopoiesis, the majority of investigators (e.g., Naegeli, '31; Lang, '38; Rohr, '40; Schiller, '43) lay great stress on the fact that myeloid transformation may occur even in cases in which the blood shows no immature cell forms. They interpret this as evidence for autochthonous formation, but the present observations do not support their claim. We have demonstrated that the immature blood cells, especially the myelocytes, hardly appear in the peripheral blood at all despite the enormous quantity of marrow elements introduced into the circulation. Cells of larger sizes such as myelocytes and megakaryocytes are arrested by the lung capillaries immediately after injection into the ear vein, and later they are gradually transported to the liver, spleen and other organs where they accumulate, without being detected again in the peripheral blood. Smaller cells such as normoblasts can pass through the lung capillaries and enter again into the peripheral circulation but only in small numbers. It is not strange, therefore, that blood examinations often fail to demonstrate the process of transport of the bone marrow elements by the blood stream even when it is really taking place on a large scale as in the present experiments.

The frequent occurrence of extravascular hematopoietic foci outside the bone marrow has been given as evidence in favor of local formation, because immature blood cells lack ameboid motility sufficient to carry them through the vascular wall (Lang, '38; Rohr, '40; Schiller, '43; and many other authors). This is not valid evidence because, in the kidney, we observed emigration of marrow elements from the arcuate vein or its cortical branches into the perivascular tissue spaces, leading to the formation of extravascular hematopoietic foci. Essentially, the process is not a true emigration but a passive change of the myeloid cell collections from an intravascular to an extravascular situation by the formation of new endothelium covering the collections and the simultaneous disintegration of the original endothelium. A similar process was observed in the incorporation of lymphocyte accumulations from the

interlobular portal vein into the periportal space of the liver (Osogoe, '50). This process is of great significance because it represents a mechanism for emigration of non-motile or slightly motile cells.

The bone marrow elements, especially the myelocytes, which accumulated in the liver, spleen and other organs, showed a large number of mitoses for 72 hours. In a later period, however, they were unable to continue to proliferate for a long time in these foreign situations when normal rabbits were used as recipients. To facilitate the further proliferation of the accumulated marrow elements outside the bone marrow, some additional factors seem to be necessary, presumably those which produce insufficiency of the hematopoietic function of the bone marrow.

CONCLUSIONS

On the basis of our results, we conclude that extramedullary hematopoiesis may occur as a result of colonization of the bone marrow elements. The question which remains to be clarified is whether a liberation of immature blood cells from the bone marrow into the circulation really takes place or not. That such liberation may occur has already been demonstrated by Livadas ('33) who used a large dose of sapotoxin as a stimulating agent. Following Livadas' methods, we are making a study of this problem, and our results so far indicate that the immature blood cells are liberated from the bone marrow on a large scale after a single large dose of sapotoxin (Ōmura and Osogoe, '49).

SUMMARY

1. A suspension of the cellular constituents of the bone marrow of the femur, tibia and humerus of two to 7 adult rabbits was given in a single injection into the ear vein of another healthy rabbit.

2. Immediately after the injection, these elements, especially myelocytes and megakaryocytes became blocked in the lung capillaries, and later they accumulated gradually in the

liver, spleen and other organs, without being seen again in the peripheral circulation with the exception of a few normoblasts.

3. From the 48th to the 72nd hour after injection, a striking accumulation of the bone marrow elements took place in the sinusoids of the liver and the red pulp of the spleen and to a lesser extent also in the small blood vessels of lymph nodes, kidneys and adrenals, with the formation of many hematopoietic foci.

4. In the kidney, small perivenous collections of myelocytes were often observed along the arcuate vein and its cortical branches where all of the successive stages of the emigration of myelocytes through the vascular wall could be traced.

5. The accumulated bone marrow elements, especially the myelocytes, showed a large number of mitoses up to the 72nd hour, but they did not continue to proliferate and disappeared to a large extent within 7 days after injection.

6. Control experiments with dead marrow elements failed to produce any foci of blood formation outside the bone marrow.

7. The results obtained give evidence for the colonization theory with regard to the histogenesis of foci of extramedullary hematopoiesis.

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EXPLANATION OF PLATES

All of the photomicrographs were taken from preparations fixed in Zenker formol and stained with Mayer's acid hemalum and eosin.

PLATE 1

EXPLANATION OF FIGURES

3 Section from the liver, showing an erythroblast collection in the markedly dilated sinusoid. Forty-eight hours after the injection. $\times 800$.

4 A diffuse collection of round cells resembling erythroblasts in the periportal space of the liver. A few typical erythroblasts (arrows) are seen in the collection. Notice the irregularly shaped and narrow lumen of the portal vein and strings of cells (St) strained across the lumen. Seventy-two hours after the injection. $\times 400$.

5 Section from the spleen, showing an aggregation of erythroblasts in a venous sinus of the red pulp. A megakaryocyte is seen below the aggregation. Forty-eight hours after the injection. $\times 800$.

6 Section from the same spleen as in figure 5, illustrating a myelocyte collection (M) in the meshes of the reticular stroma between the artificial slit spaces (Sp) of the section. $\times 800$.

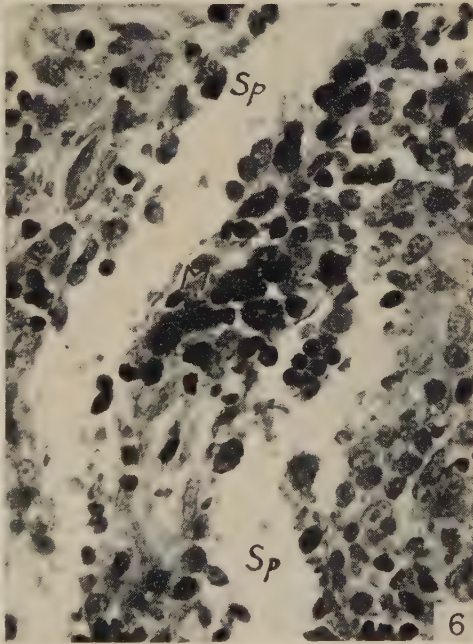
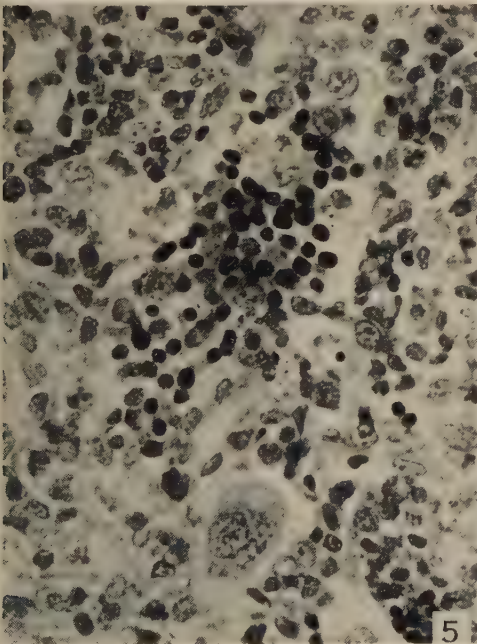
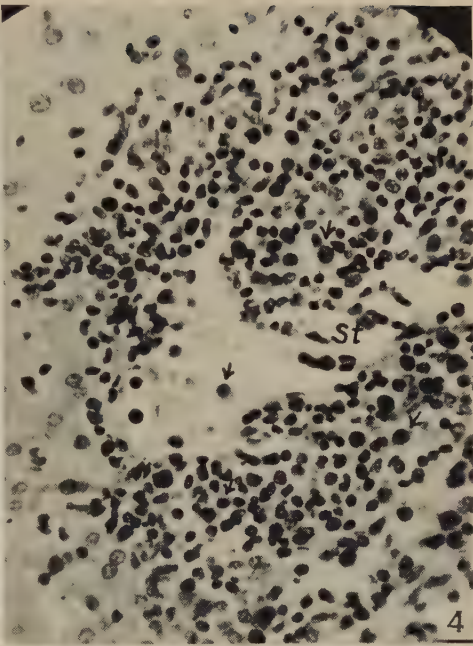
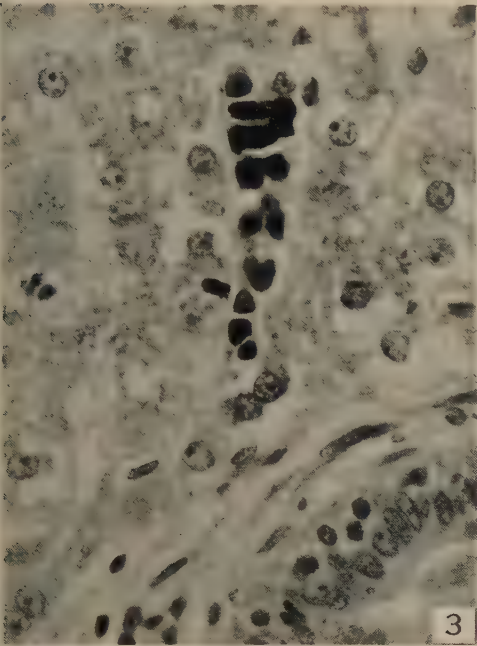


PLATE 2

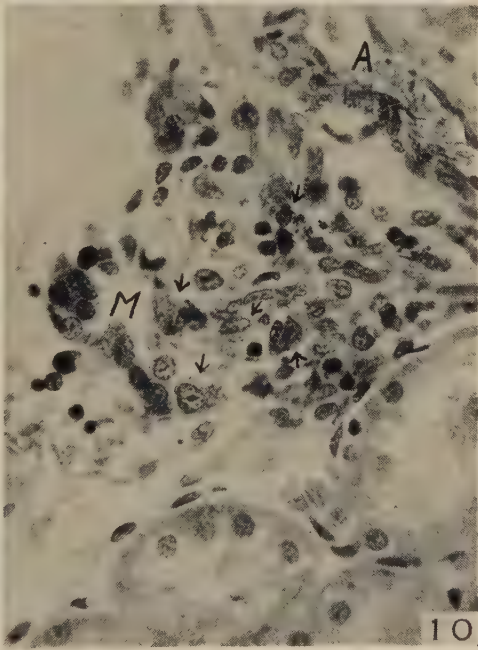
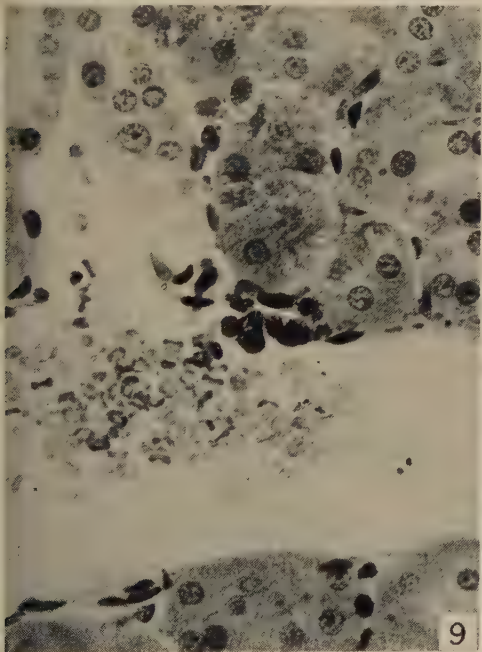
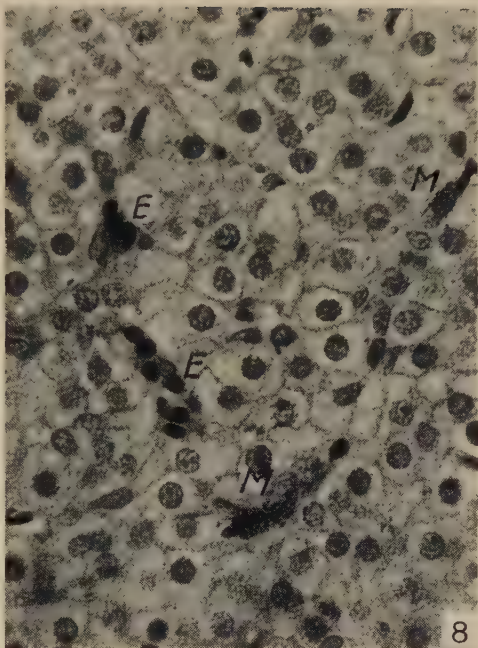
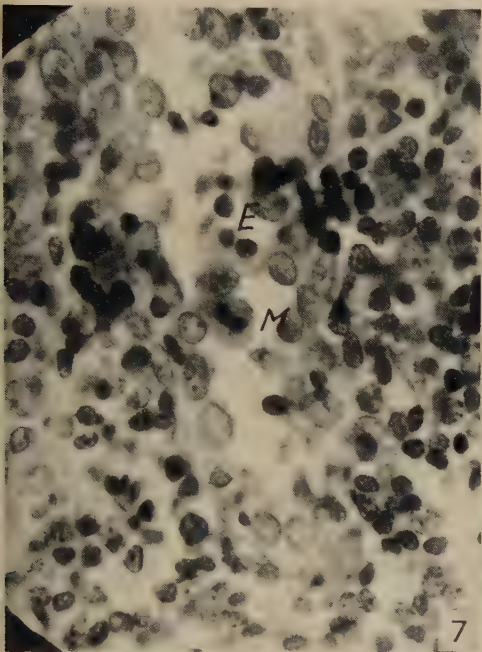
EXPLANATION OF FIGURES

7 Section through a mesenteric lymph node, illustrating the postcapillary vein including a myelocyte in mitosis (M) and three erythroblasts (E). Forty-eight hours after the injection. $\times 900$.

8 Section through the adrenal cortex. Small foci of myelocytes (M) and erythroblasts (E) are seen in the sinusoids. Forty-eight hours after the injection. $\times 800$.

9 Section from the kidney, illustrating a small myelocyte collection attached to the wall of the arcuate vein at the portion bifurcating into a cortical branch. Forty-eight hours after the injection. $\times 800$.

10 Photomicrographic illustration of the same site of the section through the kidney as drawn in figure 2. Notice some myelocytes (arrows) wandering from the myelocyte collection (M) into the perivenous connective tissue having an arteriole (A). Forty-eight hours after the injection. $\times 700$.



CHROMAFFIN TISSUE OF THE SYMPATHETIC GANGLIA AND HEART¹

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SIX FIGURES

The literature contains numerous references to the presence of chromaffin cells in the ganglia of the sympathetic system of man, other mammals, birds and reptiles.

Such statements are based upon the extensive morphological studies of Stilling (1898), Kohn ('02, '03), Wiesel ('04), Vincent ('10) and Da Costa ('34) all of whom have presented positive evidence of the existence of such cells in abdominal sympathetic ganglia. Although Vincent observed chromaffin cells in the inferior cervical ganglion of the dog, he concluded that there are very few such cells in the ganglia of the sympathetic chain.

Fulk and MacLeod ('16) demonstrated that extracts made from the chromaffin bodies of the rabbit and guinea pig yielded positive tests for adrenalin, whereas extracts of the sympathetic ganglia of these two animals yielded negative results. Gutowski ('24) later found that extracts made from the stellate ganglia of dogs and calves demonstrated the pharmacological effects of adrenalin. For a comprehensive discussion of adrenalin the reader is referred to the work of Grollman ('36), and Hartman and Brownlee ('49).

In recent years the surgical removal of lumbar and thoracic sympathetic ganglia for the relief of hypertension has provided investigators with excised yet living human ganglia. Such specimens are free of cellular autolysis, permit rapid

¹ This investigation supported (in part) by U. S. Public Health Grant H-321.

tissue fixation and offer an unusual opportunity to demonstrate the presence or absence of chromaffin cells in man. In view of the embryonic origin of the chromaffin tissue from primitive cells of the sympathetic nervous system, such a ganglionic study might also shed new light upon the origin of phaeochrome tumors.

MATERIAL AND METHODS

A total of 44 surgically removed thoracic and lumbar sympathetic trunk ganglia from 17 human hypertensive patients (30 to 59 years of age) are included in the present report. Twenty-one human ganglia were also obtained from 18 autopsy cases from 1 to 6 hours after death (29 to 80 years of age).

The fresh adrenal glands, ganglia of the sympathetic trunk, aorta and adnexal tissues of two rats, four monkeys, and one human adult provided additional control material. Sample tissues of a fatal case of bilateral phaeochromocytomata, as well as prepared sections from 4 similar tumors (courtesy Dr. A. P. Stout) were made available to the author for a cytological study of the chromaffin cells.

Following appropriate fixation (formalin, ferric chloride, potassium bichromate, Orth's fluid, mercuric chloride, chloral hydrate, chilled 80% alcohol) serial paraffin sections of the ganglia were stained with one or more of the following techniques: Bodian, Masson, benzidine, erythrosin-toluidin blue, silver nitrate, chromaffin, or alkaline phosphatase. Specific techniques used to demonstrate the "chromaffin reaction" were those recommended by Vulpian (1856), Wiesel ('02), Schmorl ('14), Wislocki ('22) and Gomori ('41, '48).

RESULTS

The adrenal glands and para-aortic tissues (rat, monkey, man), prepared with appropriate chromaffin tissue stains were first studied to discern the staining variations and histological appearance of the chromaffin cell. The most discrete chro-

maffin reactions were observed when prepared by the methods of Schmorl, Gomori, and Vulpian. The greenish pre-epinephrine granules are then easily identified in the cytoplasm of these small ovoid cells. Our best control sections were obtained from the medulla of the human adrenal gland, and we were surprised at the great variations in the shape, size and distribution of the chromaffin cells. They were often ovoid in shape and arranged in loose cords, or as alveolus-like nests of cells possessing an abundant capillary and sinusoidal network. Less frequently the cells are singly dispersed as small stellate, or large round cells engorged with cytoplasmic granules. It would often be impossible to delimit the faint outline of the cell were it not for the specific granules distributed in its cytoplasm. Similar cells were observed in the para-aortic tissues and in the aortic sympathetic nerve fibers. In the latter case however, the chromaffin cells seemed to bear a closer relation to the strands of abundant lymphoid tissue than to the nervous tissue.

Observations on the above control tissues were invaluable to us in interpreting our findings in the surgically removed sympathetic ganglia. Being aware of the pleomorphism and staining variables of the chromaffin cells, we were able to accurately identify them when they were selectively stained, as well as identify what previous investigators had erroneously designated chromaffin cells.

It is the author's opinion that earlier workers may have designated mast cells as chromaffin cells. In the sympathetic ganglia, as in other regions of the body, one encounters an unusually large number of mast cells. The latter are also pleomorphic and possess discrete metachromatic granules in their cytoplasm if toluidin blue has been incorporated in the staining procedure (M in fig. 1).

Similar difficulty will be encountered unless one is cognizant of the frequency and staining properties of the lipochrome pigments in nerve cells. Such pigment granules are sharply stained by the chromic salts and this selective staining

of granules may lead to error, especially if observed in ganglion cells of small diameter (GC in fig. 1).

A futile search was made through the entire series of hypertensive and control ganglia in an attempt to identify intraganglionic chromaffin cells. Only in one instance did we observe what appeared to even remotely resemble chromaffin cells lying in the periphery of a ganglion and partially embedded in the capsule (fig. 2). Unfortunately the specimen was not prepared to demonstrate chromaffin granules and thus the cells were not specifically stained and positively identified. These large, ovoid cells lacked the histological features of mature sympathetic ganglion cells, and in many respects resemble the primitive chromaffin cells so frequently seen in active pheochromocytomas.

Although discrete chromaffin cells were not positively observed within any of the sympathetic ganglia, we demonstrated chromaffin cells in paraganglia adjacent to the sympathetic ganglia in three different individuals. The vascularity and general appearance of a paraganglion is well shown in figure 3. With higher magnification (fig. 4) one can observe the variations in size and arrangement of the pale chromaffin cells. The nests, whorls, or alveolus-like clusters of chromaffin cells, shown in figure 4, present a histological appearance which is usually observed in pheochromocytomata. A paraganglion in which the granules of the chromaffin cells are selectively stained is shown in figure 5. The discrete cytoplasmic granules and customary vascularity of a paraganglion are well demonstrated in this preparation. In other instances the paraganglionic chromaffin elements were often small, stellate cells containing variable amounts of cytoplasmic granules.

In the course of another investigation on the conduction system of the heart, the author observed elements in the right atrium of the dog heart which appeared to be chromaffin cells. When such serial sections were selectively stained with several chromaffin techniques, a positive "chromaffin reaction" was obtained in each instance. To the authors's knowledge, chromaffin cells have never been selectively demonstrated

within the heart, and for that reason they are included in the present communication (CC in fig. 6). In figure 6, as in the adrenal medulla, the chromaffin cells vary considerably in shape, size and amount of demonstrable cytoplasmic granulation. An abundant blood supply is present in this chromaffin tissue, and leads one to wonder what role such cells and their secretions play in the control of the heart. Whether or not such cells exist in the human heart must await further investigation.

COMMENTS

We are prone to agree with the statement of Vincent ('10) that there are very few chromaffin cells in the ganglia of the sympathetic chain. Although current texts describe such cells as common elements of the sympathetic ganglia, the original investigators quoted, made their observations on the aortic plexus of sympathetic fibers — not on the ganglia of the sympathetic trunk. Chromaffin cells are to be found in the paraganglia adjacent to the sympathetic trunk *vide supra*, but these organized bodies are always outside the sympathetic ganglionic capsule and usually at some distance from it. It is not surprising that we observed sympathetic paraganglia in only three individuals, for the sympathetic trunk and ganglia are carefully dissected and cleaned during their surgical removal.

Although surgical specimens do eliminate post-mortem cell changes, their method of procurement may have been responsible for some of our negative chromaffin observations in the sympathetic ganglia. Elliott ('12), and Stewart and Rogoff ('24), previously demonstrated that anesthetic agents and manipulative stimulation of the splanchnic nerves causes a discharge and exhaustion of adrenalin. Hence, it is plausible that our failure to observe a "chromaffin reaction" in surgically removed ganglia may have resulted from a depletion of adrenalin by the anesthesia, splanchnic nerve stimulation, and ganglionic manipulation prior to appropriate fixation. Realizing that the epinephrine content of chromaffin cells is subject

to considerable normal variation, we would likewise advise the reader to heed the warning of Willis ('48), who has stressed the need of early fixation and a careful interpretation of all chromaffin reactions.

We are unable to verify the presence of chromaffin tissue in the stellate sympathetic ganglia (Vincent, '10, Gutowski, '24) since such fresh human material was not available for study. The localization of chromaffin cells in the inferior cervical ganglion and heart of man and other mammals must await further investigation.

The phaeochromocytomata reported by Miller ('24), Boyd ('26), Blacklock ('34), and Phillips ('40), implicate the ganglia of the abdominal and cervical sympathetic trunk as the probable sites of origin. From the findings reported above on thoracic and lumbar sympathetic ganglia, it is more likely that such tumors arose from the adjacent paraganglionic masses of chromaffin tissue.

SUMMARY AND CONCLUSIONS

Microscopic examination following appropriate staining failed to disclose any chromaffin cells in the thoracic or lumbar ganglia of the sympathetic trunk in man. Chromaffin tissue is demonstrable in the strands of the sympathetic plexus surrounding the abdominal aorta, and in the paraganglia lying adjacent to the sympathetic trunk. Chromaffin cells were also observed within the right atrium of the dog heart. Errors of cellular interpretation and technique have been noted.

It is suggested that the chromaffin (phaeochrome) tumors described in the literature, as associated with the sympathetic trunk, probably arose from the paraganglia.

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PLATE 1

EXPLANATION OF FIGURES

1 Selective staining of the cytoplasmic granules in mast cells (M) and of lipochrome pigment in two small ganglion cells (GC). Lumbar ganglion of hypertensive female, age 45 years. Wiesel's chromaffin stain. $\times 250$.

2 Undifferentiated cells resembling primitive chromaffin cells. Note nuclei, cell shape, and adjacent vascularity. Thoracic ganglion of hypertensive female, age 47 years. Erythrosin-toluidin blue. $\times 450$.

3 Paraganglion lying in adipose tissue adjacent to lumbar sympathetic ganglion. Note general histological appearance and vascularity. Normal male, age 35 years. Hematoxylin and eosin. $\times 100$.

4 Higher magnification from paraganglion shown in figure 3. Note arrangement and pleomorphism of the pale chromaffin cells and the intercellular stroma. The nests, whorls, and alveolus-like arrangement of chromaffin cells is similar to that observed in phaeochrome tumors. $\times 400$.

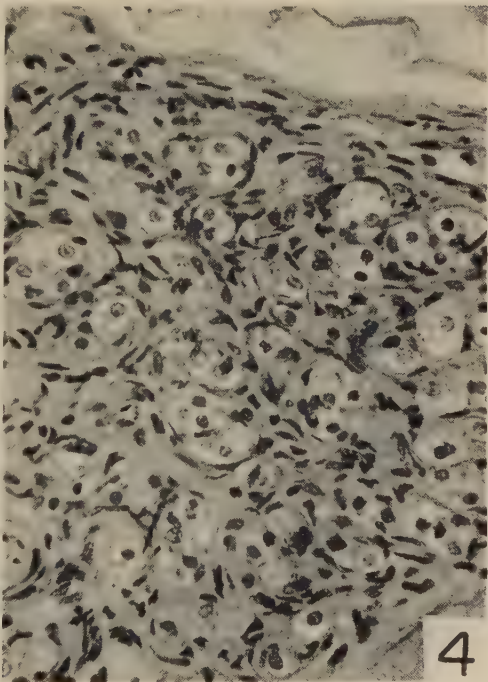
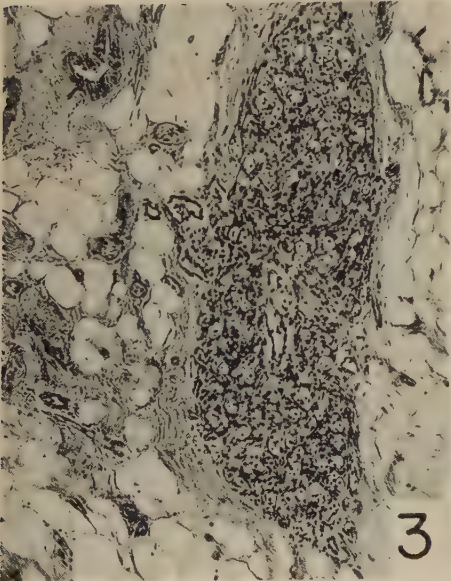
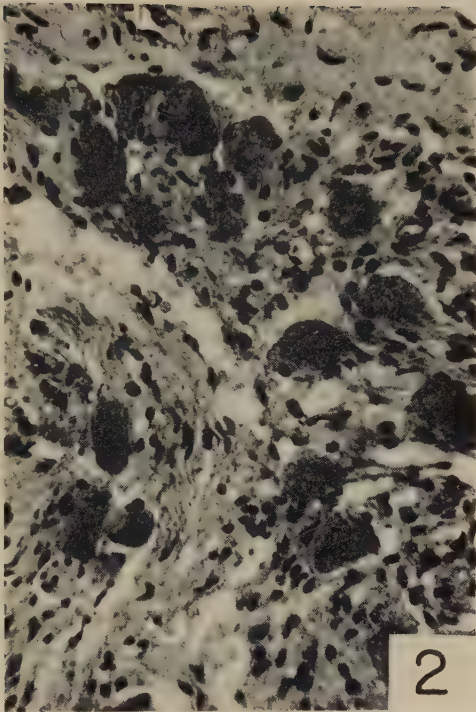
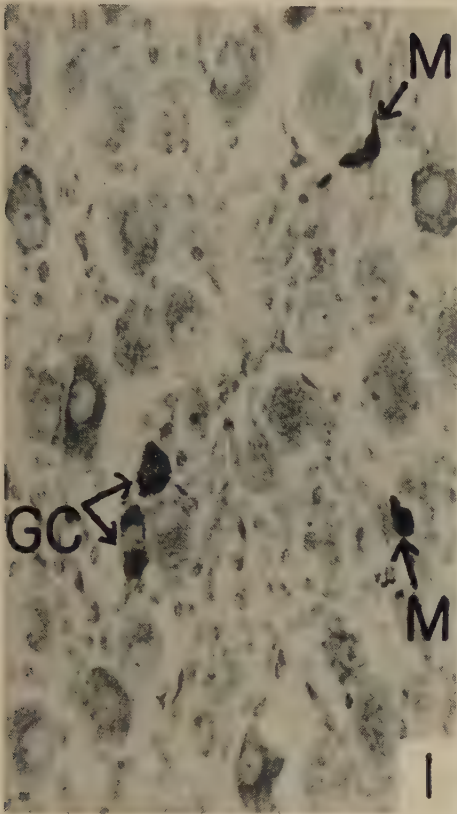
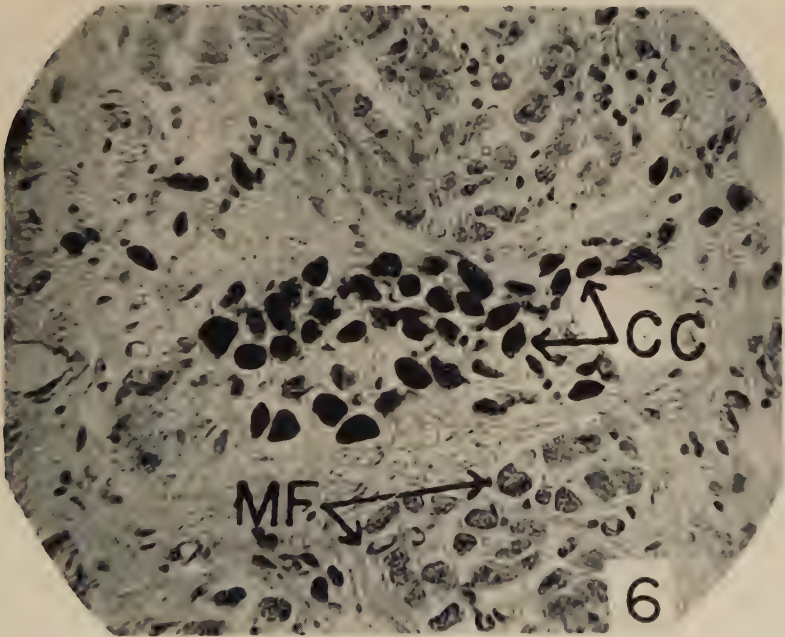
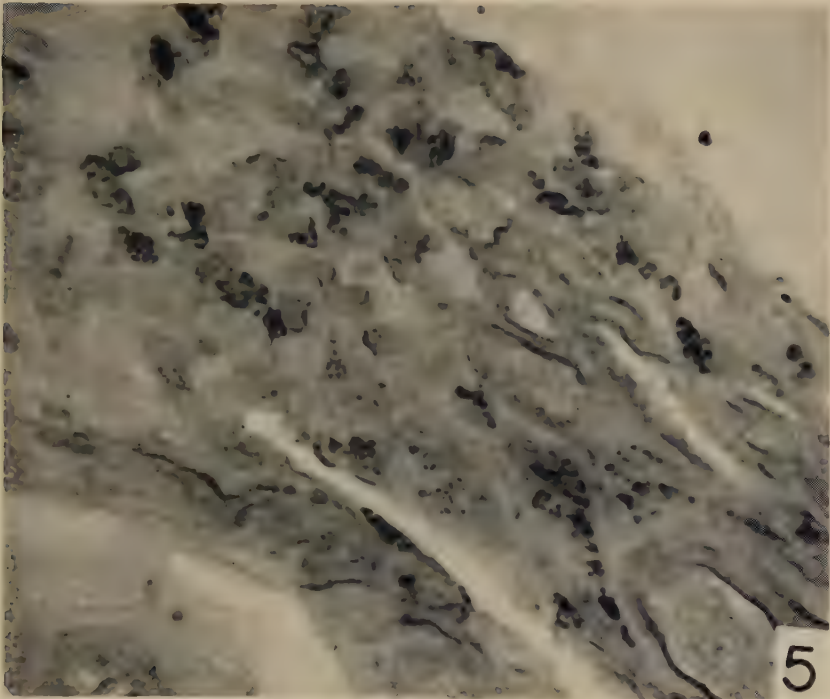


PLATE 2

EXPLANATION OF FIGURES

5 Paraganglion with selective staining of cytoplasmic granules in chromaffin cells. Note variation in size and amount of granules; the abundant blood vessels in this tissue are evident, though unstained. Lumbar ganglion of control male, age 42 years. Gomori technique. $\times 400$.

6 Chromaffin cells (CC) in right atrium immediately above the atrioventricular node. Adjacent atrial cardiac muscle fibers (MF) are more lightly stained. Note variation of cytoplasmic granulation and pleomorphism of chromaffin cells. Heart of adult male dog. Schmorl's chromaffin stain. $\times 400$.



POLYOVULAR FOLLICLES IN THE C58 STRAIN OF MICE ¹

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TWELVE FIGURES

Reports in the literature of the occurrence of polyovular follicles in the ovaries of mice are not abundant. Chappelier ('09) observed some follicles with two ova in the ovaries of white mice. Engle ('27) reported that in a series of 100 mice he found 12 ovaries in which two triovular and 16 biovular follicles were present.

While making a comparative study of the ovaries of 8 inbred strains of mice it became evident that polyovular follicles are infrequent in all with the exception of the C58 strain. Here polyovular follicles were found in all of the ovaries examined and a more detailed study seemed advisable.

MATERIALS AND METHODS

Mice of the C58 strain have a high incidence of leukemia and were extensively used for the study of this disease in the important works of MacDowell and his associates. The origin of the strain is described in MacDowell's paper: Genetic aspects of mouse leukemia ('36). The C58 strain of mice maintained in our laboratory are descendants of those that he donated to us for experimental use. The animals that are included in the present study were from the 82nd to the 84th generation of inbreeding. The right ovaries and oviducts of

¹ This investigation was supported in part by a research grant by the National Cancer Institute of the National Institute of Public Health Service.

20 virgin females of ages ranging from two weeks to 14 months were sectioned serially at 8μ and stained with hematoxylin and eosin.

OBSERVATIONS

Polyovular follicles in all phases of development were found in the ovaries of C58 mice. In the youngest follicles observed, the ova were surrounded by single layers of fusiform follicular cells (fig. 1 a). In the next stage, the follicular cells were cuboidal (fig. 1 b). Follicles that were further developed had two layers of follicular cells, each ovum being separated from them by its own zona pellucida. At this stage the biovular follicles could be divided into three types: (1) oval shaped follicles containing round, equal sized ova (fig. 7); (2) round follicles containing oval shaped equal sized ova (fig. 3); and (3) round follicles containing round ova of unequal size (fig. 8). The latter type of follicle occurred less frequently than the other two. Figure 4 shows a biovular follicle which had progressed further in development. The follicular cells were stratified and a cavity filled with liquor folliculi was present.

In larger follicles the ova were sometimes situated at different levels and could be observed only by following the serial sections. Figure 2 shows a large follicle that contained three ova; two of these may be seen in the section photographed. The relative location of the third ovum is indicated by dotted lines. Two of the ova were about equal in size, one ovum was larger and showed degenerative changes. Figure 5 is of a follicle containing 5 ova; parts of 4 of these can be seen in the section photographed. A trioovular Graafian follicle is shown in figures 9 and 10. The ovum in figure 9 is in the position normally occupied by the ovum of a monovular follicle and is surrounded by its discus proligerous, while each of the other ova has a single layer of follicular cells around it (fig. 10). Although the ova looked normal morphologically, the antrum was filled with blood which is not a normal occurrence in the Graafian follicles of mice.

Follicular involution or atresia is a common occurrence in mouse ovaries at all ages. Polyovular atretic follicles were also found in the ovaries of the C58 strain of mice. Figures 6 and 11 show biovular atretic follicles in each of which some of the follicular cells showed signs of degeneration and in each follicles one ovum was "naked" and slightly shrunken.

Occasionally atypical cell division of the ovum within the follicle takes place in mouse ovaries and may lead to the formation of several cell fragments enclosed in a single zona pellucida. A follicle containing such an atypically divided ovum is shown in figure 12 and cannot be mistaken for a polyovular follicle.

The oviducts associated with three of the ovaries contained ova. In two of the ovaries the number of newly formed corpora lutea corresponded with the number of ova present in the oviducts (4 in 1 and 2 in the other). In one oviduct there were 4 ova; one was near the ovarian end and three were in close proximity to each other farther down in the oviduct. The ovary contained only two newly formed corpora lutea. This observation indicated the possibility that of the two follicles that ruptured one liberated a single ovum, the other discharged the three closely associated ova.

Each of the 20 ovaries examined was found to contain polyovular follicles in varying number. These were counted, but because of the difficulty in following every large follicle and because some of the young follicles could not be clearly distinguished as polyovular, the count may underestimate the actual number of such follicles. Table 1 shows the ages of the mice and the number of polyovular follicles counted in the ovaries of each; the average number of polyovular follicles in the 20 ovaries was 6.1.

It may be noted that 6 ovaries from animals 3 to 5 months old had 73 polyovular follicles or an average of 12 per ovary. Four ovaries from animals younger than this had 17 polyovular follicles or an average of 4.2 per ovary. The ovaries of the 10 animals over 5 months contained 32 polyovular follicles or an average of 3.2 per ovary.

TABLE 1

LEDGER NO. OF MICE	AGE IN MONTHS	TOTAL NO. OF POLYOVULAR FOLLICLES	NUMBER OF FOLLICLES CONTAINING				
			2 ova	3 ova	4 ova	5 ova	6 ova
5764	$\frac{1}{2}$	4	4				
5746	$\frac{1}{2}$	6	6				
5032	2	4	4				
5034	2	3	3				
5094	3	8	6	1	1		
5133	3	7	6	1			
5156	4	10	9	1			
5097	4	12	11	1			
5163	5	11	11				
5754	5	25	14	7	2	1	1
5386	8	2	2				
5449	9	1	1				
5448	9	5	4			1	
5291	10	6	5		1		
5447	10	5	4	1			
5465	11	5	5				
5681	12	2	2				
5647	13	3	2	1			
5367	13	1	1				
5607	14	2	1	1			
Total	20	122	101	14	4	2	1
Average 6.1							

DISCUSSION

The ovogenesis of the mouse was carefully studied by Kingery ('17) who concluded that the definitive germ cells develop by growth and differentiation of the germinal epithelium, and the follicles by grouping of a few cells of the same origin around the growing ovum. He stated that after sexual maturity no more egg cells or follicle cells are derived from the epithelium. However, Allen ('23) has shown that ovogenesis in sexually mature mice continues the same way. He also found that: "A cyclical proliferation of the germinal epithelium gives rise to a new addition of young ova to the cortex of the adult ovary at each normal oestrous period."

The formation of polyovular follicles in the C58 mice therefore may be considered the result of atypical development and

differentiation of the germinal epithelium. As the proliferation of the germinal epithelium is correlated with the estrous cycle a hormonal effect may be an influencing factor.

The fact that polyovular follicles were present in all the ovaries examined (100%) is unique in this strain. Engle observed such follicles in only 12 of the 100 mouse ovaries that he studied (12%). Fekete ('46) in a comparative study of the ovaries of virgin dba and C57 black mice did not see any polyovular follicles. Consistent occurrence of these follicles in this highly inbred C58 strain demonstrates the importance of an hereditary factor for the formation of polyovular follicles.

Concerning the fate of polyovular follicles, Lane ('38) was of the opinion that in the rat they degenerated before the establishment of antra folliculi. In the C58 strain of mice polyovular follicles were present in all phases of their development and although many were seen in the process of degeneration some seemed normal morphologically. One indication that polyovular follicles release viable ova would be an unusually large litter size. However these mice are not exceptionally good breeders having an average litter size of 4.3 young. This figure is based on the breeding records of 26 females having a total of 89 litters and 389 young. Breeding records of course are not entirely reliable in estimating the number of ova liberated at each ovulation because they do not take into consideration the possible uterine mortality. Further studies are planned to determine more definitely whether polyovular follicles ovulate normally.

SUMMARY

In a series of 20 ovaries of virgin mice of the highly inbred C58 strain polyovular follicles were found consistently (average 6.1 per ovary). They were found to be present in all phases of follicular development.

It is suggested that the formation of these follicles may be considered the result of atypical development and differentiation of the germinal epithelium.

As the proliferation of the germinal epithelium is correlated with the estrous cycle, a hormonal effect may be a factor.

The consistent presence of polyovular follicles in this highly inbred strain shows the important influence of heredity.

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PLATE 1

EXPLANATION OF FIGURES

- 1 a and b Two young biovular follicles from the ovary of Mouse no. 5097 $\times$ 600.
- 2 A triovular follicle showing two of its three ova. The position of the third ovum is outlined with dotted lines. Mouse no. 5156 $\times$ 200.
- 3 Biovular follicles. Mouse no. 5647 $\times$ 200.
- 4 A biovular follicle with an antrum. Mouse no. 5156 $\times$ 200.
- 5 A follicle showing 4 out of its 5 ova. Mouse no. 5448 $\times$ 200.
- 6 A biovular atretic follicle. Mouse no. 5133 $\times$ 200.
- 7 Biovular follicle with round ova. Mouse no. 5156 $\times$ 200.
- 8 Biovular follicle with ova of unequal size. Mouse no. 5156 $\times$ 200.

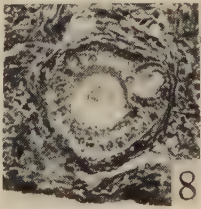
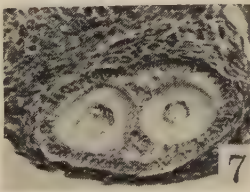
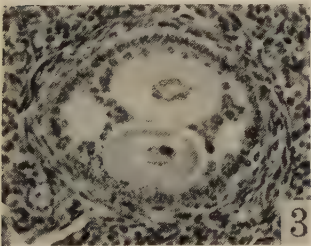
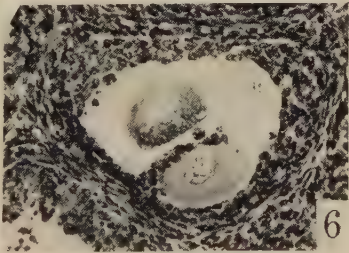
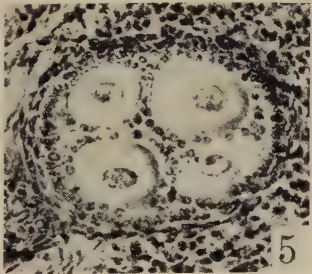
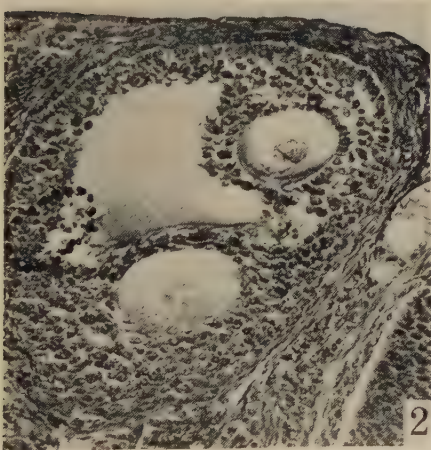
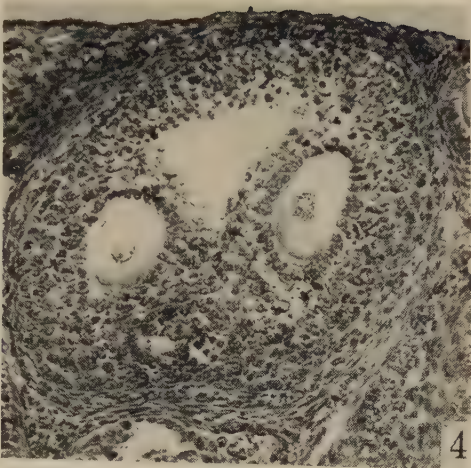


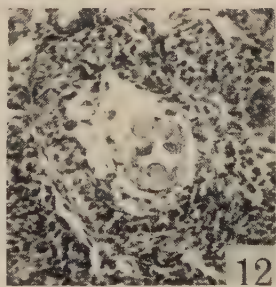
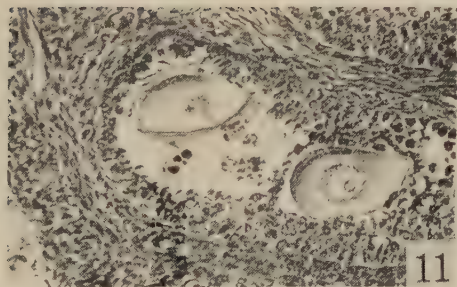
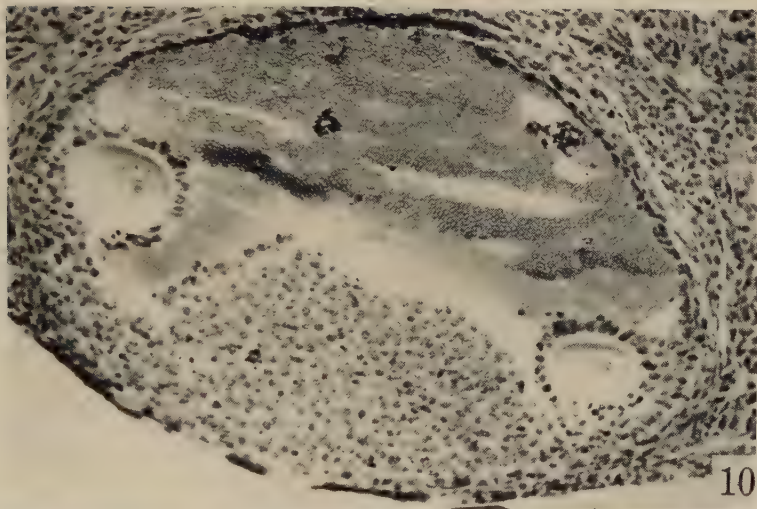
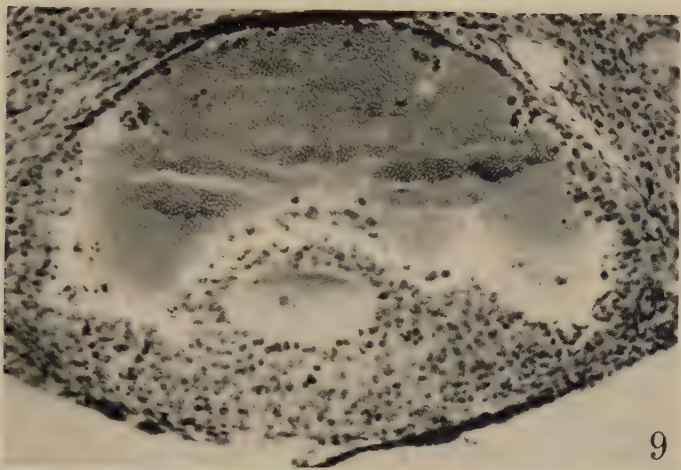
PLATE 2

EXPLANATION OF FIGURES

9 and 10 Two sections of a single triovular Graafian follicle. Mouse no. 5133 $\times$ 200.

11 A biovular atretic follicle. Mouse no. 5097 $\times$ 200.

12 A follicle containing cell fragments of an ovum enclosed in one zona pellucida. $\times$ 200.



AN ANOMALOUS LEFT CORONARY ARTERY IN A HUMAN FETUS: ITS PASSAGE THROUGH THE LEFT ATRIUM AND POSSIBLE DISCHARGE INTO THE RIGHT ATRIUM

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TWENTY-TWO FIGURES

INTRODUCTION

A marked aberration from the usual pattern of the coronary arteries was discovered by accident during a study of the blood supply to the walls of the coronary vessels of adult and fetal hearts. In one specimen, the presence of a coronary artery in the left atrium was noted. At first, this seemed incredible, but further study confirmed this fact. The examination of the literature dealing with the anomalies of coronary arteries (see Abbott, '46; Born, '34; Haferkorn, '35; Martin, 1894 and Peacock, 1858) failed to reveal cases of similar nature. This led to the careful study of all the coronary vessels of the unusual heart in the present report.

MATERIALS AND METHODS

The heart of a 22 week old fetus had been removed, injected and fixed by Dr. Benjamin N. Kropp. Knowing that I was working with the coronary arteries, he placed this material at my disposal, for which my gratitude is here expressed. Dr. Kropp states that to all appearance, the fetus was normal in development. The heart measured 31 mm in height, 18 mm in greatest lateral diameter, and 11 mm in greatest dorso-

ventral diameter. It was doubly embedded, sectioned serially at 20 μ , and stained with hematoxylin and eosin.

OBSERVATIONS

The coronary arteries in the heart of this 22 week old fetus do not all arise from two major vessels. In place of the usual left coronary artery, three vessels arise independently from the posterior aortic sinus. Two large ones we shall call the left coronary and the ventral interventricular arteries; a small one the "adventitial" artery. In place of the usual right coronary there is one large artery and two small ones arising directly from the anterior aortic sinus. None of the above arteries can be considered normal, although the right coronary approaches a normal condition. The left coronary complex is the extreme variant.

The left coronary artery arises from the left posterior aortic sinus (figs. 1, 6, and 12). It dips down slightly and almost immediately divides into two branches, which, for descriptive purposes, may be called branches A ("circumflex") and B (left marginal) (figs. 1, 5, and 17).

Branch A passes directly towards the left atrium, penetrates its wall close to the base of the septum primum and ascends on its medial wall for about 4.5 mm (figs. 1, 7, and 13). Then further branch A penetrates the wall of the left atrium close to the base of the pulmonary artery and passes toward the right atrium (figs. 1, 8, 14, 9, and 15). On reaching the right atrium, artery A passes through its wall (figs. 1, 10, and 16). As will be noted from figure 16, part of the wall of artery A is missing, probably forced off by the injection fluid. However, a few sections below and also above the level at which figure 16 is taken, the structure is intact and has the appearance of a valve (figs. 21 and 22). During the passage of branch A from the left to the right atrium, a number of small branches are given off. Most of these are muscular branches, supplying the region close to their origin. Some reach the periphery and supply the dorso-lateral region on the right side of the heart (figs. 1 and 11).

Branch B of the left coronary artery turns ventrally and laterally of the left atrium and runs downward along the left marginal surface of the heart (figs. 1 and 4-2). A number of branches are given off throughout the course of this vessel.

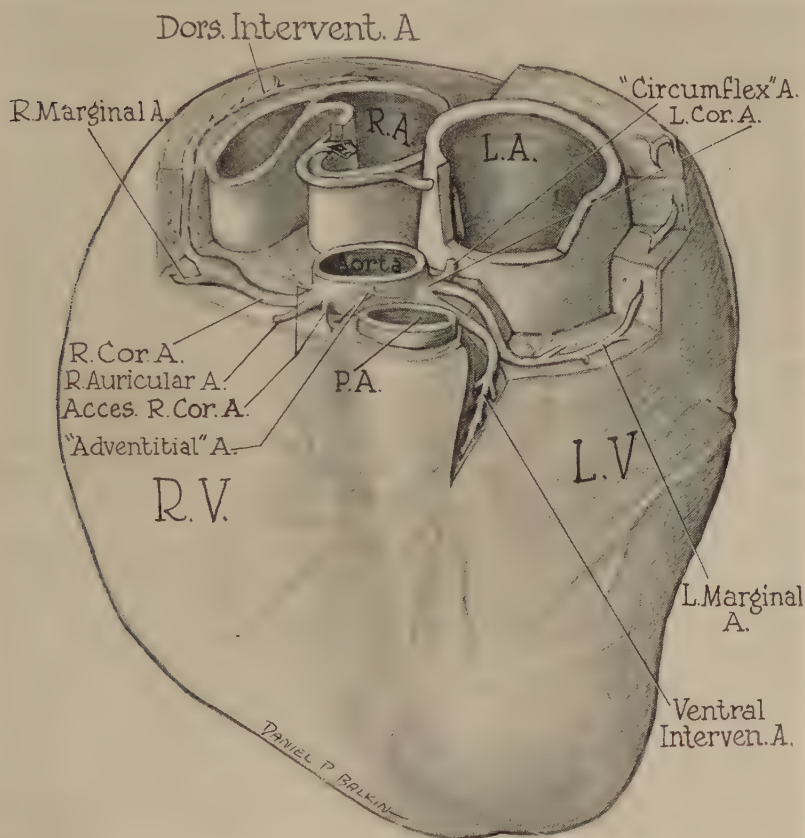


Fig. 1 Drawing based on graphic reconstruction of the heart of a 22 week fetus showing the origin and distribution of the coronary arteries.

The centrally located branches supply the root of the aorta, pulmonary artery and the wall of the left atrium and the left auricula. The peripheral branches proceed toward the apex of the heart.

In close proximity to the left coronary artery, another large vessel, the ventral interventricular artery, takes its origin from the posterior aortic sinus (figs. 1, 6, and 12). It runs downward but maintains its deeply seated position until the ventricles are reached. Then it swings ventrally and approaches the periphery. Before the periphery is reached, a number of small vessels are given off, which supply the walls of the aorta and the pulmonary artery. At the periphery, this large vessel sub-divides into three main branches (figs. 1 and 18): one occupies the ventral longitudinal sulcus (figs. 1 and 5-2) and runs to the apex of the heart, and the other two are distributed on the ventral surface of the heart, one swinging to the right and the other to the left. These do not reach the apex of the heart. A number of branches are given off. Those originating more centrally supply the median wall of the right and left ventricles. The peripheral ones are muscular branches.

A peculiar artery of small caliber, the "adventitial," originates from the ventral part of the aorta, midway between the left posterior and the anterior aortic sinuses (figs. 1, 6, and 12). Upon leaving the aorta it enters the connective tissue between the aorta and the pulmonary artery and breaks up into a number of small branches, some of which enter and supply the adventitia of the aorta and pulmonary artery. Others enter the musculature of the heart.

The *right coronary artery* is the largest artery of this specimen. It arises from the anterior aortic sinus, and soon breaks up into several branches. All of the branches are small except three, which, from their position and distribution may be called the right auricular branch, the right marginal branch, and the dorsal descending or interventricular branch (figs. 1, 5, and 19). The smaller branches of the right coronary artery are, for the most part, muscular branches, and some supply the adventitia of the aorta and the pulmonary artery. The right auricular, right marginal and dorsal interventricular arteries branch profusely, and have their normal distribution. In the region where the right mar-

ginal, the dorsal interventricular and a muscular branch take origin from the main stem of the right coronary artery, a peculiar spherical enlargement is seen. It has the appearance of a thrombus-like mass of blood cells which has caused the enlargement. The blood cells, however, appear in a fairly well preserved condition (figs. 5 and 19).

Four sections below the origin of the right coronary artery, two more arteries arise from the anterior aortic sinus (figs. 1 and 20). These are branches of small caliber, and they supply adjacent musculature of the heart.

The venous system of this heart was not studied in detail. The coronary sinus was normal and no outstanding anomalies were noted in the cardiac veins.

DISCUSSION

In the present case of anomalous coronary arteries of the 22 week old human fetus, there is very little doubt that the ventral interventricular artery is a branch of the left coronary. It is also possible that the small "adventitial" artery, although originating separately, can be included in the left coronary family of vessels. There is good reason to assume that branch A of the left coronary artery is the missing circumflex artery. Factors must have been present in its early development that interfered with its normal course of distribution. Genetic and perhaps phylogenetic factors might have been operating in the production of these anomalies. Of these, however, we have no tangible clues. The fact that the left coronary artery branches into A and B vessels as soon as it issues from the left posterior aortic sinus, instead of at the periphery, which is the normal occurrence, may well be considered as one of the major factors in this case. Because of this, and also because of its proximity to the left atrium, the circumflex artery may have become enclosed into the left atrium. Such enclosure, probably in a loop-like manner, would naturally distort its course. Consequently, in-

stead of growing peripherally, it grows centrally towards the right side of the heart. Because of mechanical imperfections at this region of the fetal heart, clear cut judgments on the further course of artery A is rendered with difficulty. Does this vessel enter the right atrium and discharge its contents there, or does it merely skirt its wall?

In favor of the latter possibility is the fact that artery A keeps its contact with the wall of the right atrium for a considerable distance before it or a branch of it proceeds to the periphery of the heart. The second possibility is supported by two concrete facts: first, the presence of a valve-like structure which is seen torn off in figure 16, but is intact in figures 21 and 22; second, the continuation of this vessel towards the periphery measures but slightly more than one-half of artery A when measured at the level of its first branching (fig. 11) (BAA).

Branch B, of the left coronary artery, which normally gives off the left marginal branch and several smaller muscular branches before it becomes the ventral interventricular artery, consists in this instance only of the left marginal artery, with its smaller branches. The ventral interventricular branch, as described above, takes a joint origin with the left coronary artery from the left dorsal aortic sinus.

The right coronary artery presents a number of anomalies. None, however, are as involved as those of the left coronary artery. The two smaller arteries arising from the ventral aortic sinus a few sections below the origin of the right coronary must be considered as branches of the right coronary artery. They are comparable to the adventitial artery seen in figures 1, 6, and 12, arising from the base of the left coronary artery. The thrombus-like mass of blood elements found at the bifurcation of the right marginal and the posterior interventricular arteries is probably not a true thrombus, but rather a postmortem blood clot, and as such could not have contributed to the death of fetus.

SUMMARY

Three separate arteries take origin from the left posterior aortic sinus: the left coronary, the ventral interventricular and a small vessel supplying the aorta and pulmonary arteries.

The left coronary artery, near its exit from the aortic sinus, divides into a descending branch, the left marginal and an ascending vessel which runs an extraordinary course. The latter approaches and enters the left atrium and ascends on the medial wall. Then it leaves the left atrium and turns toward the right atrium into which it enters and probably opens.

From the right ventral aortic sinus arise the right coronary artery and two minor vessels. The latter two supply adjacent musculature. The right coronary artery divides into the right auricular, the right marginal, and the posterior interventricular arteries. The distribution of the last three arteries falls within the limits of normal variation.

The ventral interventricular artery, although originating separately from the aortic sinus, undoubtedly represents a branch of the left coronary artery.

The ascending branch of the left coronary artery must be considered as the missing circumflex artery. The immediate subdivision of the left coronary artery into the circumflex and the left marginal artery is assumed to be the major condition for its abnormal development.

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PLATE 1

EXPLANATION OF FIGURES

- Drawings of cross sections of the fetal heart illustrating the origin and distribution of the coronary arteries. The vessels are thickened. The most cephalic level is represented by section 11.
- 2 Normal disposition of the main coronary arteries except that the ventral interventricular artery has not yet divided into its three major branches (fig. 5).
 - 3 Ventral interventricular artery leaves its centrally located position and moves peripherally.
 - 4 The passage of artery B, left marginal, to periphery of the left side of the heart. Also the branching and subbranching of the right coronary artery and its passage towards the dorsal periphery of the heart.
 - 5 Division of the left coronary artery into arteries A and B. Also origin of the right coronary artery.
 - 6 Shows the origin of the left coronary, the ventral interventricular and the adventitial arteries, also artery A (branch of the left coronary) passing through the wall of the left atrium.
 - 7 Artery A in the left atrium.
 - 8 After ascending for 4.5 mm, artery A leaves the left atrium.
 - 9 Artery A courses toward the right atrium.
 - 10 Artery A penetrates the wall of the right atrium.
 - 11 Peripheral branches of artery A.

ABBREVIATIONS

A, aorta	P, pulmonary artery
AA, artery A ("circumflex")	RA, right atrium
AB, artery B (left marginal)	RAu, right auricular artery
BAA, branches of artery A	RC, right coronary artery
DI, dorsal interventricular artery	RM, right marginal artery
LA, left atrium	RV, right ventricle
LC, left coronary artery	VI, ventral interventricular artery
LM, left marginal artery	X, adventitial artery
LV, left ventricle	Z, blood clot

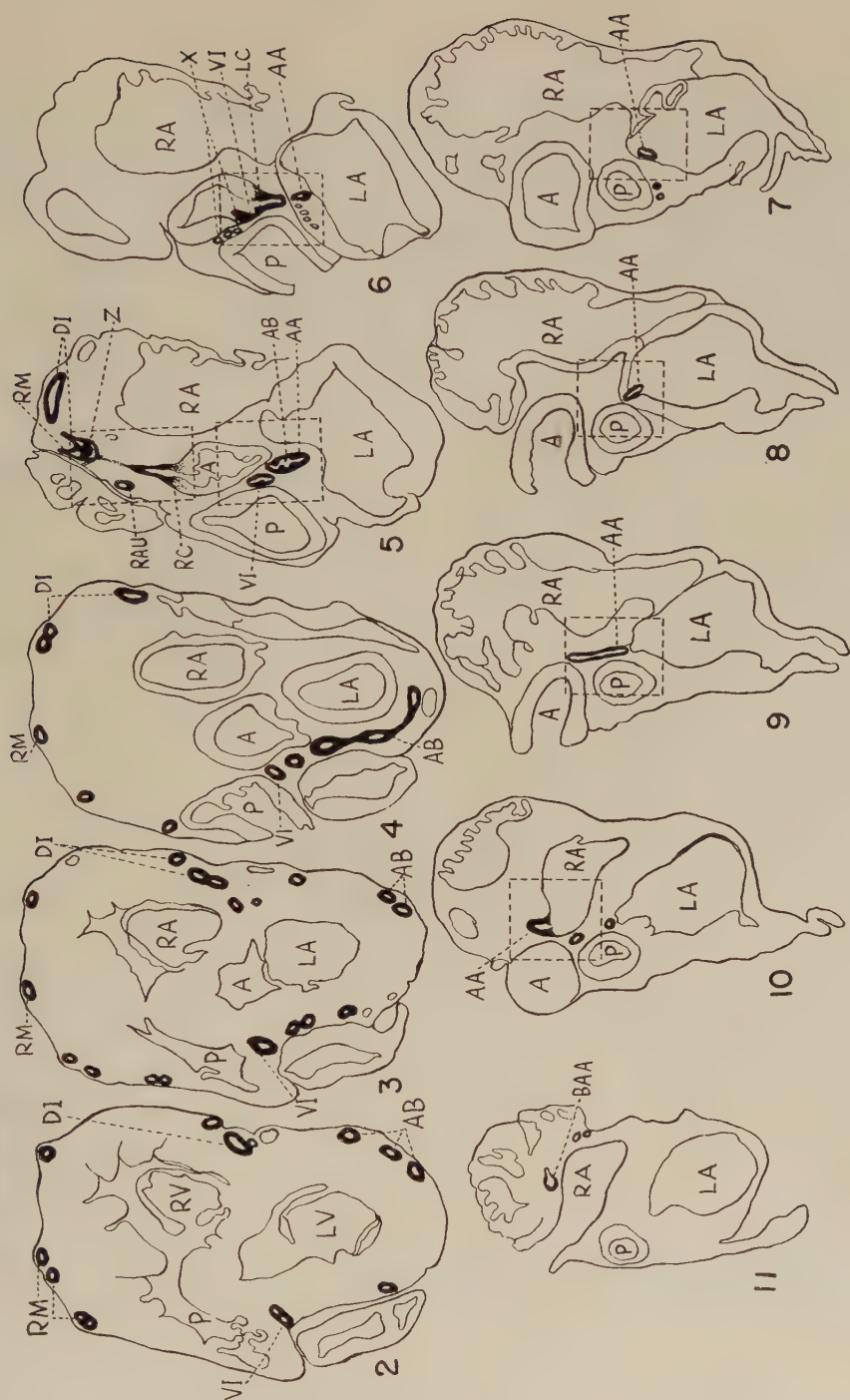


PLATE 2

EXPLANATION OF FIGURES

Photomicrographs of significant cross-section levels illustrating the position of coronary arteries.

- 12 Inset of figure 6.
- 13 Inset of figure 7.
- 14 Inset of figure 8.
- 15 Inset of figure 9.
- 16 Inset of figure 10.
- 17 Lower inset of figure 5.
- 18 The change from central to peripheral position of the ventral interventricular artery and its division into three main branches.
- 19 Origin of the right coronary artery. Upper inset of figure 5.
- 20 Origin of the accessory branches of the right coronary artery.

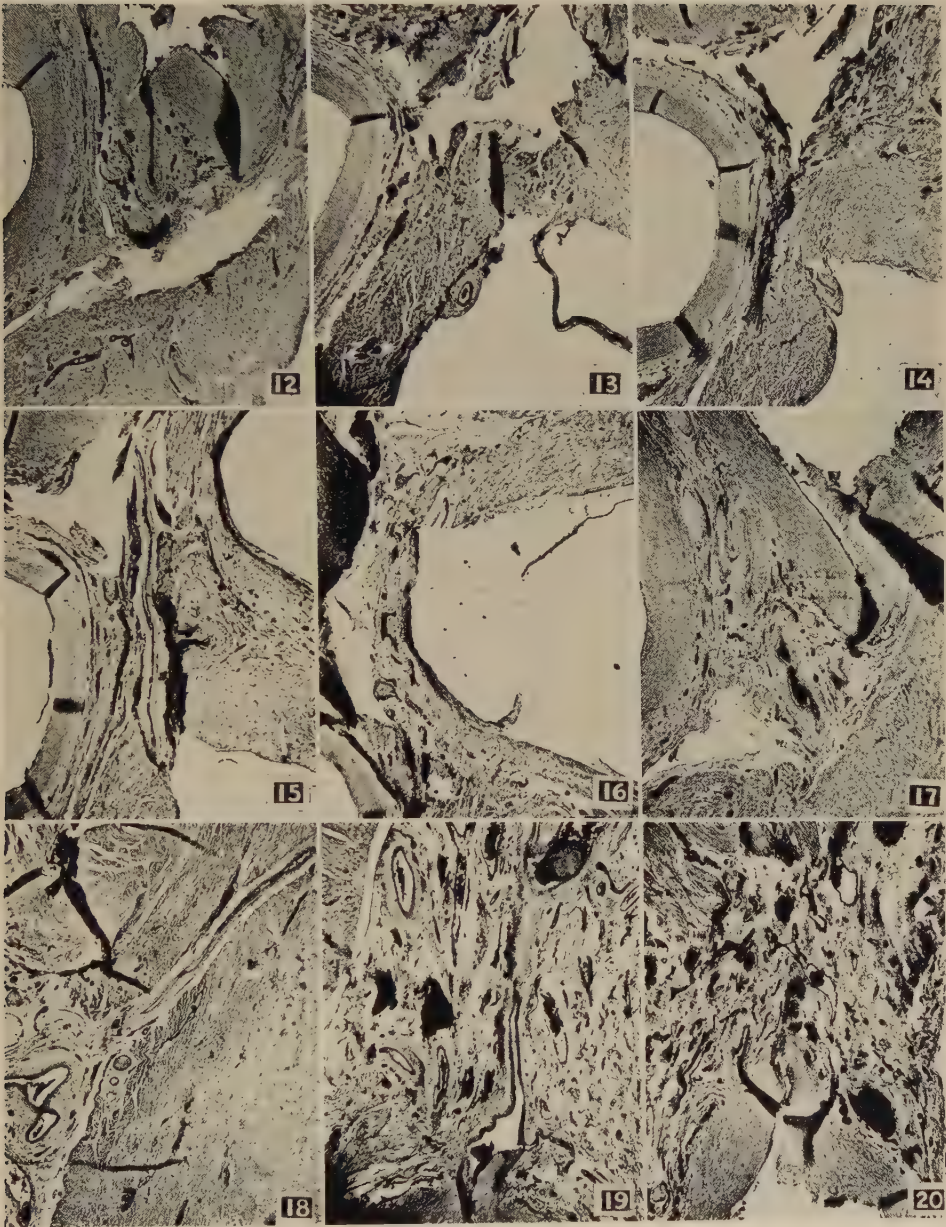
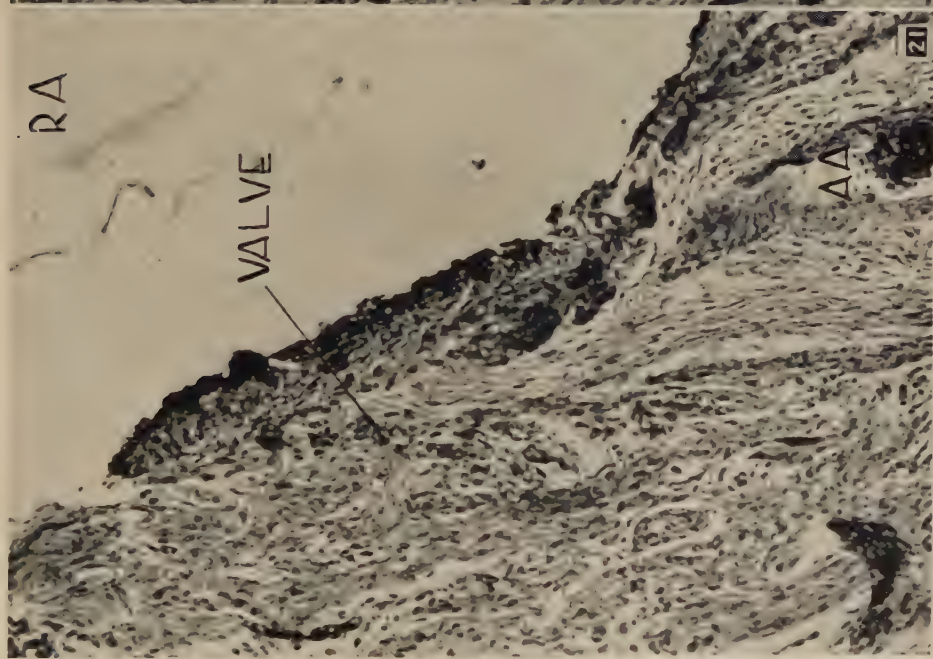


PLATE 3

EXPLANATION OF FIGURES

- 21 Artery A is seen in the wall of the right atrium. The opening of artery A into the right atrium cannot be traced at this level. Section is taken slightly above that shown in figure 16. $\times 200$.
- 22 The opening of artery A into the right atrium is traceable at this level. Section taken slightly below that of figure 16. $\times 200$.



A HISTOCHEMICAL STUDY OF STAINING THE AXIS CYLINDER WITH FUCHSIN- SULFUROUS ACID (SCHIFF'S REAGENT)¹

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SIX FIGURES

During a study of the effect of methylcholanthrene on nerves in mouse skin, the present writer ('47) found that nerves and nerve endings could be stained with Feulgen's nucleal reaction. Liang ('47), assuming "that the nervous tissue contains some reducing agent of aldehyde nature," demonstrated that nerves could be stained with Schiff's reagent without hydrolysis. Recently, McManus and co-workers ('50) observed that certain human nerve fibers were stained with the periodic acid-Schiff's reagent method, and inferred that "it may be that a carbohydrate of choline esterase type is present in some nerve fibers." Thus far it is clear that nerve elements do react with fuchsin-sulfurous acid to give a typical purple color; beyond this, however, any statement regarding the chemical nature of the substance in nerve tissue responsible for the color change, if made without more direct evidence, would be conjectural.

We must realize that fuchsin-sulfurous acid reacts with a number of substances, and that aldehydes are not the only compounds which give the typical color change. It is true that fuchsin-sulfurous acid is a sensitive reagent for alde-

¹ This study was aided by a grant (C-1180; F-1188) from the United States Public Health Service and in part by an institutional grant from the American Cancer Society to the Sloan-Kettering Institute.

² Postdoctorate research fellow of the National Cancer Institute, United States Public Health Service.

hydes; yet by no means is it also specific and selective. Bromine, for example, gives it a purple color, which in fact is utilized as one of the standard tests for this halogen (Feigl, '46). Besides, unsaturated compounds containing the carbon-carbon double bond react with fuchsin-sulfurous acid just as typically as aldehydes with the carbon-oxygen double bond (Lison, '32). Furthermore, certain aldehydes, such as vanillin, *p*-aminobenzaldehyde, and *p*-dimethylaminobenzaldehyde, do not give the typical purple color at all (Feigl, '46). In view of this lack of specificity and selectivity on the part of the fuchsin-sulfurous acid, the purple color change alone would appear to be inadequate evidence for the chemical nature of the reacting substance.

Yet, a knowledge of the precise nature of the substance in nerve tissue capable of giving the fuchsin-sulfurous acid a typical color change is undoubtedly of some significance. This will not only improve our technique in its morphological demonstration, but will also increase our understanding of its physiology. For instance, a permanent preparation of the stained specimen is difficult to obtain, thus rendering it doubtful whether the myelin sheath or the axis cylinder is stained. Had the nature of the substance been better known, we could perhaps have overcome this difficulty without much trouble. On the other hand, if "some reducing agent of aldehyde nature" or "a carbohydrate of the choline esterase type" were proved to be involved, our present knowledge of the metabolism of nerve tissue must then be duly modified. In this study, an attempt was made to determine by a series of tests the chemical nature of the substance in nerve tissue reactive with the fuchsin-sulfurous acid. Evidence is to be presented that the unsaturated fatty acid derived from lecithin is responsible for the color change.

The writer wishes to thank Dr. J. J. Bieseke for his interest, criticism and suggestions throughout this study.

MATERIAL AND METHODS

When a piece of fresh abdominal muscle or brain from mouse is treated with fuchsin-sulfurous acid, after 20 to 30 minutes the nerves are stained a purple color. However, in attempting to render the specimen a permanent mount, the stained nerves are readily decolorized in alcohol, especially of 80–90% strength. Conversely, if the fresh tissue is fixed in alcohol, the nerves are then no longer stainable, whereas the alcohol now becomes reactive with fuchsin-sulfurous acid with a typical purple color change. This could mean only one thing; that a certain substance capable of reacting with fuchsin-sulfurous acid is extractable from nerve tissue with ethyl alcohol. Advantage was taken of this shortcoming in histological technique. In this study, mouse brain was extracted with cold ethyl alcohol, and the alcoholic extract was subjected to a number of chemical tests.

Since ethyl alcohol is often contaminated with such substances as acetaldehyde, it was obviously necessary to purify the alcohol before use. This was accomplished by following the standard method as prescribed by Scott ('39) to rid the alcohol of aldehydes and other impurities. Alcohol thus purified should give no color change whatever with fuchsin-sulfurous acid. Throughout this study, only the aldehyde-free alcohol was used.

C57 strain mice were used exclusively. The animals were sacrificed by cervical dislocation. Brain tissue was freed of surrounding connective tissue, and the whole brain was extracted with aldehyde-free 90% alcohol from 24 to 48 hours. The extract was filtered to get rid of hair and other debris before performing the tests. Methods were chosen to test for the presence of (1) free bromine, (2) compounds with carbonyl linkage and (3) compounds with ethylenic linkage. For economy of space, detailed procedures of some methods are not reproduced here, but reference to the origin of each method is always indicated.

EXPERIMENTS AND OBSERVATIONS

1. *Fuchsin-sulfurous acid test.* Fuchsin-sulfurous acid was prepared according to an improved method described by Coleman ('38). To 2 ml of the acid in a test tube, 0.5 ml of the alcoholic extract was added carefully so the latter floated on top of the reagent. The extract soon lost its transparency and changed its color from the original straw color to white. After about 20 to 30 minutes, there appeared in the alcoholic layer three distinctive color zones; the upper red, the middle purple and the lower white. After shaking, the alcoholic layer mixed well with the reagent, and the mixture then appeared reddish purple. If this mixture was left standing for about 12 hours, a precipitate formed floating in the mixture. The precipitate was of a typical purple, while the mixture showed a reddish color. By adding 0.1 to 0.2 ml of NHCl , the red color was discharged, while the purple color of the precipitate remained unaffected. Thus, according to some authors (Oster and Mulinos, '44; Oster and Oster, '46), the red color of the mixture was a "pseudoreaction," while the purple color of the precipitate was a "true reaction." It is noteworthy that the lapse of time (20 to 30 minutes) for the appearance of the purple color was about the same in the extract as that of the nerve tissue. This is in sharp contrast to the instantaneous appearance of purple color when bromine or some aldehydes were tested (see below).

2. *Separation and classification of compounds.* There is the possibility that alcohol might extract from mouse brain a mixture of compounds. To effect their separation, the extract was freed from alcohol by aeration. The residue was dissolved in ether and the scheme for separation of a mixture of water insoluble compounds as listed by Schriner and Fuson ('44) was followed. After separation, each fraction was tested with fuchsin-sulfurous acid. Solution 2 gave a negative reaction; it was, therefore, discarded. Solution 4 gave a "pseudoreaction" and ether layer 4 gave a "true reaction." Therefore, in the extract, the substance capable of generating

a purple color with fuchsin-sulfurous acid belonged to the class of neutral compounds.

To confirm this finding, the ether from ether layer 4 was evaporated. Residue from ether layer 4 and residue from the original alcoholic extract were subjected to the solubility tests. Both were found to be insoluble in water, NaOH and HCl; soluble in sulfuric acid but insoluble in syrupy phosphoric acid. Therefore, the substance belonged to Class N₂ of neutral compounds, which includes all aldehydes, alcohols, ketones and esters containing more than 9 carbon atoms, quinones, ethers and unsaturated hydrocarbons.

3. *Tests for free bromine.* Though it appears rather unlikely that free bromine is present in nerve tissue, yet a theory of sleep was proposed by Zondek and Bier (Best and Taylor, '45) based on the liberation of a bromine compound from the hypophysis. Thus the possibility cannot be dismissed too lightly. The following tests were performed to determine whether or not free bromine is present in nerve tissue.

When 0.5 ml of 1% bromine water was added to 2 ml of fuchsin-sulfurous acid, a purple color appeared instantaneously. Even bromine vapor, when brought in contact with the reagent, caused the color to change. If left standing, a purple colored precipitate formed. The formation of precipitate could be brought about by adding more bromine. The precipitate was insoluble in water, alcohol and most other fat solvents. No red color was evident. On adding N HCl, however, the solution gradually became red, and concurrently the purple colored precipitate disappeared. In these respects, the precipitate produced by bromine behaved quite differently from that produced by the alcoholic extract of nerve tissue (see above).

The alcoholic extract of nerve tissue was further tested for free bromine by the fluorescein test (Feigl, '46) and the alpha-naphthoflavone test (Feigl, '46). Both tests gave negative results. From these results and the differences in the behavior of the precipitates, it is concluded that in the al-

coholic extract of nerve tissue, the substance which reacts with fuchsin-sulfurous acid is not free bromine.

4. *Tests for carbonyl compounds.* A number of aldehydes and ketones were tested with fuchsin-sulfurous acid. Results are summarized in table 1. The following points are worthy of notice. (A) Most aldehydes gave an instantaneous color change. Generally, water insoluble aldehydes required a longer time. (B) Not all aldehydes gave the typical purple color. Chloral gave a pink color; 2,4-dihydroxybenzaldehyde gave a brown color; *p*-dimethylaminobenzaldehyde gave an orange color and vanillin gave a yellow color. (C) Addition of NHCl did not affect the color change of aldehydes. (D) Some ketones reacted with fuchsin-sulfurous acid to give a typical purple color. (E) All ketones required a longer time for the color to change than aldehydes. (F) Addition of HCl discharged the purple color of methyl *n*-amyl ketone, but had no effect on the purple color of ethyl butyl ketone. It is evident, therefore, that fuchsin-sulfurous acid is not specific for aldehydes, because it reacts with some ketones and with bromine. Furthermore, fuchsin-sulfurous acid is not selective for aldehydes, because some aldehydes do not give the characteristic purple color.

On the assumption that the substance in the alcoholic extract reactive with fuchsin-sulfurous acid is a carbonyl compound, it will be expedient to know more of its identity. Attempts were made to obtain addition products and solid derivatives. If a solid derivative were obtainable, then it would be feasible to determine its physical constants and perhaps its chemical composition. Unfortunately, considerable difficulties were met with and the results are far from convincing.

To purify the compound, the alcoholic extract was added to a saturated aqueous solution of sodium bisulfite. After thorough mixing and vigorous shaking, no heat production was noticeable and no crystalline addition product was evident. After cooling and addition of more alcohol, a voluminous white precipitate formed. The precipitate was collected and

dissolved in water. After adding 10% HCl, the solution was extracted with ether. When tested with fuchsin-sulfurous acid, the ether extract gave no color change at all. Repeated tests gave consistent negative result.

TABLE 1

Different color changes between aldehydes and ketones with Schiff's reagent

NAME OF COMPOUNDS	REACTION TIME	COLOR CHANGE	HCL EFFECT
Acetaldehyde	quick	purple	none
Benzaldehyde	slow	purple	none
Bromal	quick	purple	none
<i>n</i> -Butyraldehyde	quick	purple	none
Chloral	quick	pink	none
Decyl aldehyde	quick	purple	none
2,4-Dihydroxybenzaldehyde	slow	brown	yellow
<i>p</i> -Dimethylaminobenzaldehyde	slow	orange	none
Formaldehyde	quick	purple	none
Furfural	quick	deep red	none
<i>n</i> -Heptaldehyde	quick	purple	none
2-Hydroxy-5-chlorobenzaldehyde	slow	deep red	red
<i>m</i> -Nitrobenzaldehyde	slow	deep red	none
Nonyl aldehyde	quick	purple	none
Phenylacetaldehyde	quick	purple	none
Salicylaldehyde	quick	brown-black	brown
Tolyl aldehyde	quick	purple	none
Vanillin	slow	yellow	none
Acetone	none	none	none
<i>p</i> -Aminoacetophenone	none	none	none
Chloretone	none	none	none
Ethyl butyl ketone	slow	purple	none
Mesitylene	slow	purple	discharged
Methyl <i>n</i> -amyl ketone	slow	purple	discharged
Methyl naphthyl ketone	none	none	none

Semicarbazide hydrochloride (Schriner, '44), hydroxylamine hydrochloride (Schriner, '44) and phenylhydrazine (Schriner, '44) were used in attempting to obtain solid derivatives from the alcoholic extract. Each test was carried out as for water insoluble compounds. None of these gave a solid derivative with the alcoholic extract. Benzaldehyde was tested simultaneously as control.

Dimethylcyclohexanedione (dimethyldihydroresorcinol) was also used. This compound is proved to form solid derivatives specifically with aldehydes. One method described by Weinberger ('31) and another by Schneider ('46) were closely followed. Control tests were carried out with benzaldehyde, which formed a crystalline precipitate insoluble in alcohol. No such crystalline precipitate was obtainable with the alcoholic extract.

Color tests other than the fuchsin-sulfurous acid test were resorted to for the detection of aldehyde in the alcoholic extract. *o*-Dianisidine (Feigl, '46) gave an orange color with benzaldehyde, but no color change was noticeable with the alcoholic extract. Oxidation of *p*-phenylenediamine is catalytically accelerated by aldehydes (Feigl, '46). An instantaneous color change took place when an aldehyde was added to this reagent. Formaldehyde gave a green black, acetaldehyde a green black and benzaldehyde a yellow color. Such catalytic action was not demonstrable with the alcoholic extract when added to this reagent.

It is necessary to point out that none of these tests involved a higher temperature than that of the laboratory. This point will be further elucidated. From the tests so far performed, it is perhaps justifiable to conclude that a carbonyl compound is not present in the alcoholic extract of nerve tissue.

5. *Tests for ethylenic compounds.* Oleic, linoleic and linolenic acids were chosen to represent unsaturated fatty acids with one, two and three double bonds. To three test tubes, each containing 2 ml of fuchsin-sulfurous acid, 0.5 ml of 1% alcoholic solution of each of the acids was added carefully so that the acid floated on top of the reagent. Soon the acids became translucent and the region close to the reagent appeared as a colloidal emulsion. After about 5 minutes, a red color appeared in the alcoholic layer. After standing for about an hour, the red color near the white opaque layer began to change from red to purple. By this time, there were three distinctive color zones: the upper red, the middle purple and

the lower white. When the mixtures were left standing for about 24 hours, a purple oily precipitate formed, floating in the mixture. Addition of N HCl discharged the red color but left the purple color unaltered. The reaction of these unsaturated fatty acids with fuchsin-sulfurous acid appeared very similar to that of the alcoholic extract of nerve tissue as described in 1.

On the basis of the above observation, experiments were set up to isolate the unsaturated fatty acid from the alcoholic extract of nerve tissue. To 40 ml of the alcoholic extract, 20 gm of potassium hydroxide in 60 ml of water was added. The resulting mixture contained 20% of potassium hydroxide in 40% alcohol. The mixture was warmed on a water bath until saponification was complete. It was then diluted with 100 ml of water and again heated on a water bath to drive off the alcohol. Hydrochloric acid was added until the mixture was acid to litmus. Fatty acids, which rose to the surface, were collected. When these fatty acids were tested with fuchsin-sulfurous acid, a typical purple color developed.

The fatty acids thus obtained were further purified according to the low temperature solvent crystallization method described by Brown and Shinowara ('37). The fatty acids were dissolved in acetone. The acetone solution was cooled in alcohol previously brought to a temperature of -20°C . with dry ice. At the end of 12 hours, a waxy precipitate formed in the acetone solution. The waxy precipitate was filtered off. The acetone solution was further cooled at a temperature of -70°C . The crystals formed at this temperature were collected and allowed to melt at room temperature. A clear oily substance, which soon became amber colored in air, was obtained. Fuchsin-sulfurous acid test was carried out on both fractions. The waxy precipitate obtained at -20°C . gave no reaction, whereas the oily substance obtained at -70°C . gave an intense purple color.

These two fractions were further tested with Baeyer's test and bromine test. The oily substance obtained at -70°C . was capable of reducing potassium permanganate to manganese

dioxide and of decolorizing bromine in carbon tetrachloride. This capacity was not manifested by the waxy precipitate obtained at -20°C . Therefore, the oily substance was an unsaturated fatty acid and the waxy precipitate a saturated fatty acid. It is the unsaturated fatty acid that is able to react with fuchsin-sulfurous acid to generate a typical purple color. Reaction between the unsaturated fatty acid and Ludwig-Haupt reagent (Merck Index, '40) gave a yellow color, thus indicating that the acid belonged to the oleic acid series ($\text{C}_n\text{H}_{2n-2}\text{O}_2$). Further identification of the acid was deferred until a later study.

6. *Tests for lecithin.* From what has been described so far, it appeared very likely that a phospholipid was extracted from nerve tissue by alcohol. Among the phospholipids, lecithin is known as being readily extractable with ethyl alcohol in the cold. A solubility test was performed on the residue from the alcoholic extract, after the alcohol was evaporated by aeration. It was found to be soluble in chloroform, ether, benzene, carbon tetrachloride, carbon disulfide and petroleum ether, but insoluble in water, acetone and methyl acetate. Consequently, the residue was dissolved in ether, and the ether was poured into twice its volume of acetone. A voluminous precipitate formed. When examined with the microscope, the precipitate showed typical myelin figures in water or in saline solution (fig. 1).

There is always a possibility that lecithin is contaminated with cephalin. To further purify the lecithin, an improved method described by Pangborn ('41) was adopted. Cadmium chloride was used to precipitate out lecithin from the alcoholic extract. This was repeated several times until the alcohol became non-reactive to fuchsin-sulfurous acid. The cadmium compound was washed with alcohol and then with ether. It was then suspended in petroleum ether and extracted with 80% alcohol. The petroleum ether was freed in vacuo and the precipitate collected. The precipitate was suspended in chloroform and a cold 25% solution of ammonia in methyl alcohol

added. After being concentrated on a water bath, the lecithin was collected with ether.

The purified lecithin was further identified by the following tests. (A) it reduced osmic acid to form a black precipitate. (B) It reacted with Casanova's reagent (Merck Index, '40) to form a reddish ring which changed to green and then to intense blue. (C) It reacted with alloxan to give a pink color (Merck Index, '40). (D) It dissolved in sulfuric acid resulting in a yellow solution which became purple red on the addition of sugar (Merck Index, '40).

Interesting phenomena were observed when the purified lecithin was treated with fuchsin-sulfurous acid. A drop of lecithin in ether solution was placed on a cover slip. After evaporation of the ether, a drop of water was added to allow the formation of myelin figures. After about 10 to 15 minutes, a drop of fuchsin-sulfurous acid was added, and the cover slip was mounted on a slide. Under the microscope, the myelin figures were observed to change color gradually from yellow to pink, red and finally purple. What is so interesting is the fact that not all of the myelin figure showed the color change, but only the central core was deeply stained, while the peripheral region remained colorless. The stained myelin figure appeared very similar to a peripheral nerve fiber with its axis cylinder and myelin sheath, save for the presence of node of Ranvier and cell of Schwann (fig. 2).

Examination with Nicol's prisms revealed that the stained centrum was isotropic and the colorless periphery was anisotropic (fig. 3). This led the present writer to re-examine the above-obtained two fractions of fatty acids with polarized light. It was found that the unsaturated fatty acid was isotropic, while the saturated fatty acid was anisotropic (fig. 4).

A freshly dissected out peripheral nerve fiber was examined with crossed Nicols. The axis cylinder was observed to be isotropic while the myelin sheath was anisotropic (fig. 5). Based on these observations, it is perhaps reasonable to believe that at least part of the myelin sheath is composed of

saturated fatty acid, and part of the axis cylinder of unsaturated fatty acid.

7. *Tests to render stained specimen permanent.* Once the substance in nerve tissue responsible for the color change of fuchsin-sulfurous acid was known to be an unsaturated fatty acid derived from lecithin, it appeared possible to find a means to render the stained nerve a permanent mount. A number of compounds had been tried. Finally, hydrobromic acid and picric acid were found to possess this quality.

A piece of fresh abdominal muscle was treated with fuchsin-sulfurous acid. After the nerves were well-stained, the tissue was washed in sulfurous acid followed by hydrochloric acid to rid the tissue of any excess fuchsin-sulfurous acid. At this stage, the stained nerves were decolorizable with alcohol. If the tissue was immersed in 1% hydrobromic acid for 12 hours, or for the same length of time in a saturated solution of picric acid, the stained nerves were rendered alcohol-resistant. Thereafter, the tissue could be dehydrated with alcohol and mounted in diaphane or through xylol in Canada balsam. An examination under the oil immersion lens revealed that the axis cylinders were stained. No change so far is noticeable in specimens prepared in this manner 6 months ago (fig. 6). However, the hydrobromic acid must be applied after the nerves are stained; otherwise, the nerves are then no longer stainable. In this respect, picric acid has definite advantages over hydrobromic acid, because the former can be used before or after the staining. The picric acid can be washed out completely with hot water or 1% HCl at 60°C., or be left in the tissue as a counterstain.

DISCUSSION

It is well-known there are two different Feulgen reactions: the so-called "nuclear" and the "plasmal." The "nuclear reaction" generally takes place in the nucleus. It requires acid hydrolysis. The stain is resistant to alcohol. The "plasmal reaction" generally occurs in the cytoplasm. In this case, acid hydrolysis is unnecessary, but the resulting color is read-

ily extracted by alcohol and other fat solvents. An earlier observation by the present writer ('47) that nerves and nerve endings can be stained with fuchsin-sulfurous acid after acid hydrolysis of material fixed in formalin-picric acid alcohol (Rossman's fluid), appears to be a "nucleal reaction," while Liang's ('47) finding that nerves and their endings can be stained with the same reagent without hydrolysis indicates it is a "plasmal reaction."

Feulgen and Voit ('24) considered that the substance in the cytoplasm reactive with fuchsin-sulfurous acid was "lipoid" in nature. It exists in the cytoplasm in the form of "plasmalogen." The "plasmalogen" itself does not react with fuchsin-sulfurous acid. It is converted by treatment with mercuric chloride or dilute acids (HCl , H_2SO_3) into "plasmal," which gives the fuchsin-sulfurous acid instantaneously a definite purple color. Feulgen and Behrens ('38) were able to work out the chemical constitution of "plasmalogen." It is similar to ester phosphatides only instead of fatty acid residue, a high aldehyde (palmitic or stearic aldehyde) is bound to two free hydroxyls of glycerophosphoric choline ester as in acetals; therefore the name acetal phosphatide, of which the high aldehyde is responsible for the "plasmal reaction."

While there is little doubt about the existence of acetal phosphatide *in vitro*, results of the present study, however, do not seem to support the idea that such also exists in nerve tissue *in vivo*. Of all the tests so far performed in this study, with the single exception of fuchsin-sulfurous acid, none has given a positive result for aldehyde in the alcoholic brain extract. Could it be that aldehyde is not present in nerve tissue under physiological conditions, but is artificially made to appear?

In all methods for the demonstration of acetal phosphatide of animal tissue, heat is invariably involved in one step or another. Feulgen ('24) suggested coagulation of tissue with hot water followed by extraction with alcohol at room temperature. He also used steam distillation to separate the

aldehyde from other lipids. Anchel and Waelsch ('42) used boiling alcohol for extraction. Klenk ('44) dissolved dried, powdered glycerophosphatide from ether extract of human brain in hot benzene and heated the mixture for one hour. Anchel and Waelsch ('42) obtained solid derivative by refluxing the alcoholic extract with *p*-carboxyphenylhydrazine or carboxymethoxylamine for two hours.

It is generally accepted that unsaturated fatty acids change their nature when oxidized. This can be affected either by autooxidation or by some oxidizing agents. Ralston ('48) summarizes excellently our present knowledge of the mechanism and products of these oxidative reactions. Suffice it to point out here that aldehydes are often formed from unsaturated fatty acids as a result of autooxidation. Moreover, this autooxidation can either be promoted by heat, light, metallic salts and aromatic nitrogen compounds; or it can be inhibited by phenolic compounds, tocopherol, gossypol and lecithin. Schibsted ('32) quantitatively determined the fat aldehydes resulting from oxidation of fats and oils, and concluded that these aldehydes are glyceride aldehydes formed when unsaturated acid radicals of fat are oxidized. He also noted that heat greatly accelerates the formation of aldehydes. The higher the temperature and the longer the heating, the greater is the amount of aldehydes formed.

It was with this in mind that tests for aldehydes involving higher temperature than that of the laboratory were purposely excluded in this study. The cold alcoholic extract gave negative results with sodium bisulfite, semicarbazide hydrochloride, hydroxylamine hydrochloride, phenylhydrazine and dimethylcyclohexanedione. On the other hand, when the alcoholic extract was heated and then subjected to these tests, reaction products were easily obtained. Hydrazone was also obtained by heating and refluxing the alcoholic extract with 2,4-dinitrophenylhydrazine. These results show that the alcoholic extract does not contain aldehyde. The aldehyde is made to appear by heat, which accelerates the autooxidation process in the unsaturated fatty acid radical in lecithin.

Feulgen and co-workers, during their studies, had insisted on the wide distribution of "plasmalogen." Verne ('29), repeating the experiments in a systematic way, arrived at the conclusion that the "plasmalogen" is not as widespread as Feulgen had insisted. The substance that gives the reaction is strictly localized. A large number of cellular elements give no reaction at all (blood cells, lymphoid tissues, thymus, glomerular epithelium, liver), in other instances the reaction is weak and diffuse (muscle, thyroid, pancreas, intestinal epithelium) and in still others the reaction is selective and intense (renal tubules, myelin sheath, small pulmonary cells, adrenal gland, corpus luteum).

In another study Verne ('41) noted that there exists between the "plasmal reaction" and lipid reaction (sudan III, etc.) a relation in space and in time. A large number of tissue fats passes through three stages. In the first stage, they give only the lipid reaction (hepatic cells, adrenal glomerulose cells). In the second stage the lipid reaction and the "plasmal reaction" exist concurrently (muscle, myelin, sheath). In the final stage, only the "plasmal reaction" is demonstrable (kidney, corpus luteum, small pulmonary cells). Verne further noted that in vivo such tissues as hepatic cells and adrenal glomerulosa cells never go beyond the first stage, but the "plasmal reaction" can be brought out artificially by using oxidants such as potassium permanganate, hydrogen peroxide, etc. Lison ('36) wrote that the aldehydic compounds revealed by the "plasmal reaction" are only products of physiological oxidation of fat substances, probably unsaturated. In vivo, such aldehydic compounds do not exist in the free state. They are "masked," by virtue of a fragile combination with an unrecognized substance, and are liberated by treatment with sublimate or dilute acids.

In his study on the specificity of Schiff's reagent for aldehyde, Lison ('32) tested in vitro 475 different compounds with different functional groups. He found that unsaturated fatty acids react exactly the same as do aldehydes. He went further to postulate that the mechanism of reaction is similar

in the two cases, i.e., ethylenic groups add to the amino groups of the dye in the same manner as do the carbonyl groups. Though this sounds very attractive, yet the possibility that the unsaturated fatty acids are changed to aldehydes by oxidation in the presence of Schiff's reagent is worth investigating.

The sulfurous acid used in the preparation of, and the excess of it left in the Schiff's reagent certainly play an important part. Budde ('09) was the first to point out that sulfurous acid can hydrolyze fats and oils into fatty acids and glycerine. In a series of papers, Wardlaw and others ('20; '22; '23; '26) demonstrated the oxidizing properties of sulfurous acid. That sulfurous acid is able to induce oxidation is well illustrated by the reaction when green hydrated nickel oxide is oxidized to form black hydrated nickel oxide (Feigl, '49).

It is generally accepted that oxidation of fats, oils and unsaturated fatty acids takes place in two well-defined stages. In the first stage, peroxides are formed. The second stage leads to the formation of hydroxy acids, hydroxy-keto acids, unsaturated hydroxy acids, lactones, and various condensation products, and finally by cleavage to aldehydes, aldehyde acids, and mono- and dicarboxylic acids. Oxidation inhibitors function mainly by prolonging the first stage, while accelerators, as well as the peroxides formed in the first stage, may catalyze or induce the oxidation to proceed to the endpoint of the second stage.

Lecithin is known to possess antioxidative properties (Ralston, '48). In vivo, oxidation processes of nerve tissue probably seldom go beyond the stage of peroxide formation. Freshly purified lecithin readily turns brown on exposure to air, presumably because of autooxidation of its unsaturated fatty acid radical. When lecithin is allowed to react with fuchsin-sulfurous acid, it is likely that the sulfurous acid first hydrolyzes the lecithin into fatty acids and other radicals, then it induces the oxidation of the unsaturated fatty acid to form peroxides, which alone or together with the excess

sulfurous acid may induce or catalyze the oxidation to proceed to the formation of aldehydes. The aldehyde formed in turn reacts with leuco-basic fuchsin to give a purple color. This perhaps accounts for the time lapse (20 to 30 minutes) between the addition of lecithin to the fuchsin-sulfurous acid and the appearance of the typical purple color. It may also account for the above-described three distinctive color zones when the alcoholic extract is added to fuchsin-sulfurous acid; the upper red, the middle purple and the lower white. The white zone may indicate where hydrolysis and oxidation are taking place, the red zone where peroxide is formed and the purple zone where aldehyde is formed.

The so-called Schiff's reagent can be prepared in a number of ways. Basic fuchsin or *p*-rosaniline decolorized by ammonium hydroxide (Feigl, '46) or anhydrous sodium sulfite (Feigl, '46) do not react with lecithin at all. Only Schiff's reagent prepared with sulfurous acid or bisulfites is capable of giving a purple color when reacting with nerve tissue or its extract. To illustrate this point, the following experiment was performed. A 0.1% solution of basic fuchsin was added drop by drop into a 1% solution of anhydrous sodium sulfite until a definite red color remained. To this mixture, enough 1% solution of anhydrous sodium sulfite was added until the red color was again completely discharged. This reagent reacts with aldehyde to give a purple color. On the other hand, when nerve tissue or its extract was treated with this reagent, no color change took place. However, when the nerve tissue or its extract was treated first with sulfurous acid for 12 hours, thoroughly washed in water and then allowed to react with this reagent, a purple color appeared almost instantaneously. Therefore, sulfurous acid is essential to bring out the typical color change when nerve tissue or its extract is to react with Schiff's reagent. Whether or not sulfurous acid actually possesses the above-suggested functions, i.e., hydrolysis, oxidation and induced oxidation, can be confirmed only by further experiments.

Pangborn ('41) used fuchsin-sulfurous acid to test the lecithin purified by her own method. She concluded that "all lecithin preparation from beef heart gave strongly positive tests for plasmalogen by the technique of Feulgen, Inhauser, and Behrens, no matter what method of purification was used." In the opinion of the present writer, her method is definitely an improvement over the one described by Levene ('27) both in regard to convenience and purity. The purified lecithin is not contaminated with acetal phosphatide, rather the latter is but a chemical artifact produced by the fuchsin-sulfurous acid.

It is surprising to note that most investigators used Schiff's reagent alone as a criterion to determine the presence and even quantity of acetal phosphatide. Since Schiff's reagent is neither specific nor selective for aldehydes, and since it reacts with unsaturated fatty acid and bromine, a positive reaction merely indicates that one of these substances is present in a certain compound. Only by employing supplementary tests is it possible to distinguish the one from the other.

Because aldehydes react with Schiff's reagent, Feulgen ('24), Verne ('29) and Liang ('47) warned that formalin must not be used as a fixative. Lison ('36), however, pointed out that such precaution is quite unwarranted. Formalin does not interfere with the reaction. It is merely necessary to wash the section thoroughly in distilled water before performing the test. In my previous study ('47), formalin-picric acid alcohol (Rossman's fluid) was used to fix the tissue. It is now comprehensible that the picric acid renders the lecithin insoluble in alcohol. It is only necessary to wash out the picric acid and formalin in warm water or HCl before staining, and thereafter the specimen may be made a permanent mount.

The fact that nerves can be stained with Schiff's reagent has been noted perhaps as early as the "plasmal reaction" was first proposed. Most observers (Verne, '29; Lison, '36) believe that the myelin sheath is stained. In this study, observations of permanently mounted specimens were made with

the oil immersion lens and of freshly stained material with the polarizing microscope. Contrary to general belief, it is the axis cylinder that is stained.

SUMMARY AND CONCLUSIONS

1. Fuchsin-sulfurous acid is specific neither for aldehydic compounds, ethylenic compounds nor free bromine. To distinguish these from each other, supplementary tests must be used.

2. When an alcoholic extract of mouse brain tissue was submitted to a number of chemical tests,⁴ it was found that the unsaturated fatty acid radical of lecithin was responsible for the color change of fuchsin-sulfurous acid.

3. Results of this study do not support the idea that an aldehydic compound is present in nerve tissue *in vivo*. *In vitro*, an aldehydic compound can be derived from unsaturated fatty acid by oxidants or by oxidation accelerators (sulfurous acid, heat, etc.).

4. The possibility is suggested that sulfurous acid plays an important role in the hydrolysis of lecithin into its several radicals, and in further oxidation of the unsaturated fatty acid to form peroxide and aldehyde.

5. Microscopic studies with both transmitted and polarized light lead to the belief that the unsaturated fatty acid is largely located in the axis cylinder, while the saturated fatty acid is largely in the myelin sheath.

6. Hydrobromic acid and picric acid are found to render the stained nerve resistant to alcoholic decoloration and thus to facilitate the preparation of permanent mounts.

7. Contrary to general belief, observation with high magnification of permanent mounts, checked by that with Nicol's prisms, reveals not the myelin sheath, but the axis cylinder to be stained.

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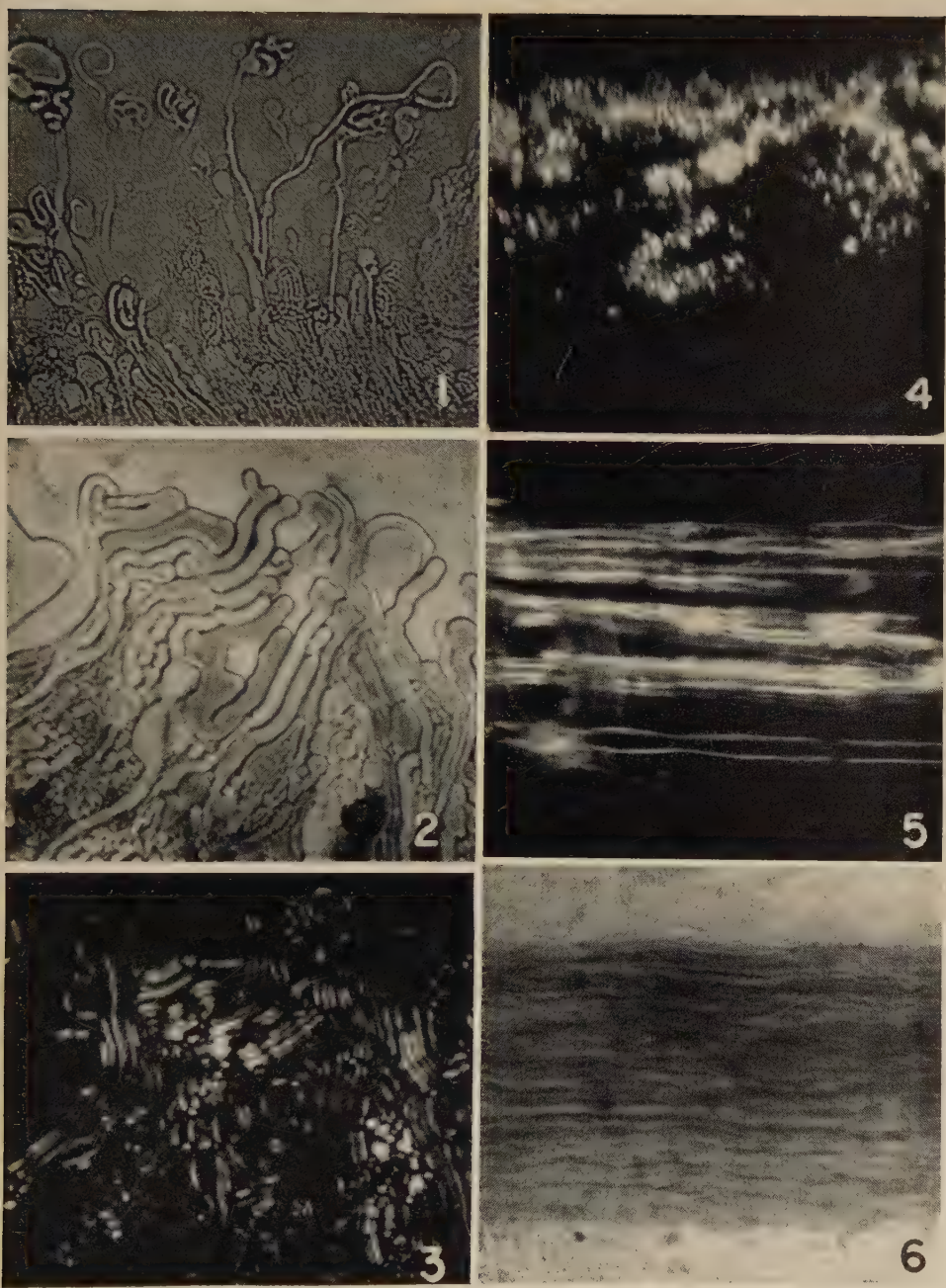
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PLATE 1

EXPLANATION OF FIGURES

- 1 Purified lecithin in normal saline solution showing formation of myelin figures. $\times 185$.
- 2 Purified lecithin stained with Schiff's reagent showing the stained centrum and colorless periphery of myelin figures. $\times 300$.
- 3 Same as figure 2 examined with polarized light showing birefringent periphery of myelin figures. $\times 300$.
- 4 Isolated saturated fatty acid fraction of lecithin examined with polarized light showing anisotropism. $\times 185$.
- 5 Fresh sciatic nerve from C57 mouse in saline solution examined with polarized light showing anisotropism of myelin sheath. $\times 300$.
- 6 A nerve trunk in abdominal muscle from C57 mouse stained with Schiff's reagent to show that the axis cylinder is stained. $\times 300$.



STUDY, RECONSTRUCTION AND GROSS DISSECTION OF THE ATRIOVENTRICULAR CONDUCTING SYSTEM OF THE DOG HEART

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FIVE FIGURES

The pathway for conduction of the cardiac impulse is of great importance in the interpretation of electrocardiograms, and a knowledge of the distribution of conducting tissue in experimental animals is imperative to research. Recent reports questioning the existence of specialized tissue, the so-called atrioventricular conducting system, in the human and canine hearts (Glomset and Glomset, '40; Glomset and Birge, '45) have necessitated the reexamination of this tissue. This study on the dog was designed to review the histological features and relationship of the system, to determine if sufficient continuity were demonstrable to permit reconstruction in wax of the atrioventricular bundle and its main branches, and to ascertain if gross dissection of the system were possible in fresh dog hearts.

MATERIALS AND METHODS

A detailed histological study was carried out on serial sections of the septum of the heart of a canine pup. Ten micra frontal sections, cut from back to front, were stained by a modification of the Masson trichrome technique (Goldner, '38), with which connective tissue stains bright green,

¹ Costs of the study were borne by a grant from the Life Insurance Medical Research Fund.

myocardium brick red, and the specialized tissue a purplish gray, at least two divisions grayer on the chroma scale of the Munsell System of Color Notation than ordinary heart muscle. Criteria for the identification of specialized tissue involved the differential staining reaction, the relative paucity of myofibrils with a tendency toward a rather clear perinuclear area when compared with ordinary myocardium, the presence of numerous fairly uniform, rounded nuclei, an intimate association with connective tissue, and continuity with similar cells.

Each section was studied microscopically, then projected and drawn at a magnification of 25 diameters. Outlines of the specialized tissue from every 4th section were cut in beeswax, with details being added from the intervening sections when necessary. Areas where undoubted end to end transitions to muscle were observed were colored with red ink. The wax plates were stacked as they were cut, orientation being derived from the superimposed outlines of the whole septum, viewed by transmitted light. The reconstruction was checked frequently to insure proper dimensions, supports were devised to prevent sagging, and fusion was finally accomplished with hot beeswax (fig. 1).

Further studies on the same heart involved the nerves of the septum associated with the A.V. node and bundle; every section was studied, projected, and drawn. The stain used was not specific for nerves. Ganglia, nerve cells and bundles of nerve fibers could be recognized, however, individual nerve fiber terminations could not be distinguished. No reconstruction of these nerves has yet been attempted.

Finally, gross dissection of the node, bundle, and primary branches was accomplished in the hearts of 7 adult mongrel dogs. These were either studied within an hour after death by asphyxia, or frozen within half an hour after death, stored in a deep freezer, and thawed rapidly in running water immediately before dissection. Both ventricles were cut open on the lateral aspect, care being taken not to disturb the papillary muscles or false tendons. Dissections of the bundle

were made most easily by working back from the right limb whenever it was visible on the surface of the ventricular septum, removing the median cusp of the tricuspid valve to facilitate dissection. In the left ventricle, a cut was made through the septal cusp of the mitral valve and the lateral wall of the aorta to reveal the septum, aortic valves, and left branch. Scale drawings were made from these specimens.

OBSERVATIONS

Microscopic study and reconstruction

The atrioventricular nodal area was located at the posterior border of the interatrial septum, fibers of the node being continuous with the atrial musculature in many areas. This nodal region varied in width from about 1.4 to 1.7 mm and extended ventrally for approximately 1.6 mm. Strands of connective tissue penetrated the node increasing in later sections as the nodal area narrowed and became concentrated in the right half of the septum. The atrioventricular bundle, as it passed through the lower part of the membranous septum, was entirely ensheathed by connective tissue. From this point on, as far as the system was studied, connective tissue was always found forming a sheath around the specialized tissue. The space between the specialized tissue and its sheath was very small in contradistinction to that found in the ungulate heart.

The upper part of the ventricular septum was narrow and shifted to the right so that the bundle came to lie at the upper border of the ventricular musculature on the left side of the septum, with a small projection of ventricular muscle to the right of it. At this point it was posterior to the membranous septum, and the first fibers of the left branch of the bundle were seen extending subendocardially down the septum; as may be seen from the reconstruction (fig. 1), the left branch was given off as a broad, thin sheet extending for approximately 2.4 mm along the bundle. About 1.8 mm from the anterior limits of the origin of the left branch, the right

branch emerged and ran as a discrete bundle embedded in the septal musculature until it reached the subendocardial surface about halfway down the septum.

The fibers of the nodal area showed the staining differentiation already mentioned, and the fibrils appeared relatively delicate, were not so prevalent as in atrial muscle, and had a greater tendency to arrange themselves at the periphery of the fibers, thus giving rise to a rather clear perinuclear area; some faint striations were visible. Nuclei stained dark, were of a relatively uniform size, round to slightly elliptical although not completely regular in outline, and appeared somewhat crowded. The fibers were arranged in an interwoven meshwork in which individual fiber continuity was obscured; this same pattern was apparent in the bundle proper. As the left branch extended down the septum, the fibers became more parallel in arrangement, the nuclei were larger and somewhat less dense than formerly, more fibrils and clearer peripheral striations were visible, and clear perinuclear areas were still to be seen. Still more fibrils and striations characterized the right branch, but the tissue was distinguished from ventricular muscle by its delicate connective tissue sheath, the presence of fewer long oval nuclei than were seen in ordinary cardiac muscle, and the differential staining reaction which was retained, although less striking than in the other parts of the system.

An artery from the posterior descending branch of the right coronary artery, after traversing the septal base, ran forward along the top of the node, passed into its substance and bifurcated, one branch passing through the center of the node into the central fibrous body and entering the ventricle, the other running in the right portion of the node and upper part of the bundle, finally passing into the ventricular septum also. These are represented by small plastic tubes on the reconstruction (fig. 2).

It is noteworthy that a nodal branch of specialized tissue was found along the caudal border of a "tongue" of atrial muscle extending to the base of the median cusp of the tri-

cuspid valve, and another nodal branch penetrated the central fibrous body to reach the connective tissue at the base of the septal cusp of the mitral valve. In addition, several small branches were traced from the right and central portions of the node and upper region of the bundle, through the central fibrous body, until they underwent end to end transition to ventricular muscle. Transitional areas are indicated by dark tips on the projections of the reconstruction (fig. 2), and the histological features of a transitional area are shown in figure 3. Direct continuity of specialized tissue with ordinary cardiac muscle was also observed in the region of the upper left branch, but was not seen in the portion of the right branch studied.

Relation of nerves to the atrioventricular system

In so far as nervous elements were identifiable in the preparations used, no large nerves approached the vicinity of the node or bundle, and no cell bodies were present in the node, bundle, or its main branches. Nerve cells were readily distinguished in the interatrial septum; small nerve tracts, presumably parasympathetic in nature, ramified through the atrial muscle close to the A.V. junction. The possibility of fine nerves and individual fibers entering the specialized tissue is by no means precluded.

Large nerves were found ventral to the nodal area, associated with the aortic wall and could be seen intimately associated with the right coronary artery in its origin and initial ramifications and accompanying the larger septal blood vessels, particularly in the lower half of the septum.

Gross dissections

Of the 7 adult dog hearts, only the right and left branches were dissected in one, since valvular vegetation obscured the region of the bundle proper; the bundles in 5 were dissected from the right side and in one, from the left side.

Heart no. 6, one of the largest specimens, will be described and compared with the other hearts studied (fig. 4). The atrial connection appeared as a bifurcated area of light pink tissue joining the atrial musculature about 5 mm in front of the opening of the coronary sinus. This bifurcation was visible in two other hearts also (hearts 3 and 7). From this junctional region a column of muscle about 1 mm in diameter and 15 mm long extended through the connective tissue of the central fibrous body to run along the top of the ventricular septum, surrounded by the connective tissue and a cartilage of the central fibrous body (heart no. 4 showed 2 small septal branches from the bundle in this region). The bundle then turned rather sharply to run along the ventricular side of the membranous septum for about 7 mm. A thin soft layer of muscle fibers and connective tissue covered the bundle and origin of the right branch in this region, as it did in hearts 3, 4, and 7 also. The right branch (in 6) continued as a discrete bundle to the base of the anterior papillary muscle, giving off fibers to this muscle and crossing the ventricular cavity to the lateral wall. At its origin the right limb was unusually broad, about 3 mm in diameter, and showed a definite change in direction from the bundle. In hearts 2, 5, and 7 there was no such abrupt change in direction, but the right branch seemed more a continuation of the main bundle. This branch was lost in the ventricular muscle in heart 3, and arose so close to the left side of the septum in heart 7, that the bundle was dissected from the left.

The origin of the left branch was diffuse (fig. 5), the bundle giving off thin slips of specialized tissue for a distance of from 5 to 9 mm in various specimens. The branch then fanned out subendocardially, extending forward on the septum, at first as a broad grayish sheet with the fibers somewhat more concentrated at the edges in most cases, then forming anastomosing bands of fibers, some of which made stellate junctions with each other. Fibers from the anteroventral concentration led to the anterior papillary muscle and those to the posterior papillary muscle usually followed two paths, one set of fibers

crossing the ventricular cavity to the apex of the muscle, another part of the same tract continuing down the septum to the base of the papillary muscle. Septal bands were visible almost to the apical portion of the septum.

DISCUSSION

The technique of reconstruction is particularly valuable in confirming the presence of a tissue in a particular anatomical relationship; in so far as it has been possible to determine, the atrio-ventricular conducting system in the dog has not previously been reconstructed. Excluding all tissues which did not fit the established criteria, there was still no loss in continuity of the specialized tissue in the septum. Study of every section also made it possible to observe transitions of specialized tissue to cardiac muscle both in the atria and ventricles, transitions which because of their daintiness might have been overlooked without a complete study, since some were limited to a few sections. In the nodal area, contrary to the reports of the Glomsets ('40), junctions of nodal tissue with atrial muscle were observed microscopically, and the bundle was followed in gross dissection to the muscular portion of the interatrial septum. No direct tracts of specialized tissue between the S.A. and A.V. nodes were seen, in agreement with Keith and Mackenzie ('10) for mammals and contrary to the situation in birds (Davies, '30).

The fact that we observed a greater tissue distinction between specialized tissue and heart muscle than was recorded by the Glomsets undoubtedly lies in the staining technique used and the differentiation obtainable by various methods. In addition, sections were observed in which a staining artifact in ventricular muscle produced a staining reaction similar to that of specialized tissue, but other cellular and histological features prevented any confusion of this tissue with conducting tissue. Other areas were observed in which connective tissue entered into intimate relationship with ordinary heart muscle, but again, the resulting effect was not that of specialized tissue. Hence it would seem that

the specialized tissue studied is an entity, with histological characteristics which cannot be explained merely as ordinary cardiac muscle subjected to staining artifacts or admixture with connective tissue.

Connective tissue in appreciable amounts, although admittedly less than in the bovine heart, was found interposed between the specialized tissue and cardiac muscle throughout the portion of the system studied, except in those areas where transition to muscle occurred. Thus, while the specialized tissue of the dog heart exhibits some differences from that of the ungulate heart, this study indicates that there are specimens in which a well defined atrioventricular system occupies a homologous position to that in the ungulates, and that it possesses those features which might argue for physiological import in both groups of animals.

His (1893) mentioned a branch of his bundle leading to the base of the aortic leaf of the mitral valve in mammals; Davies ('30) also described valvular connections to which he assigns a function in birds; we have found branches to the bases of the valves in the dog. Since the system is supposed to be neomorphic in birds and mammals and absent in poikilotherms (Davies and Francis, '46), it is interesting to speculate on the homology of these valvular connections, and to raise the question as to whether the primitive mammalian ancestors also possessed a rapid heart rate and muscular valves which contracted early in ventricular systole as is the case in birds.

Little attention has been paid to the smaller branches leading from the A.V. node and bundle to the ventricular muscle, although they have been noted in various species by Curran ('09), Lloyd ('30), Davies ('31), Robb and Kaylor ('45), Mahaim ('47), and Robb, Kaylor and Turman ('48). In the dog they were small and particularly numerous entering the posterior septum. Curran ('09) regarded the small branch that he observed as an accessory septal branch, necessary to supply the posterior muscles of the heart; Robb, Kaylor and Turman ('48) indicated that such early septal connections might influence electrocardiographic interpretation, espe-

cially since these would not be injured when the "right bundle" is cut. Mahaim ('47) used the term "paraspecific conduction" for the function of these fibers as well as those of Kent ('14), indicating that their greatest importance lay in conditions where the larger branches were functionally impaired. The recent observations of Pruitt ('50), who obtained normal EKG's in perfused hearts in which the subendocardial network was damaged by caustic, probably represent instances where these early, relatively protected branches, as well as the deep septal branches, transmit the cardiac impulse to the myocardial network of specialized fibers.

The present findings are in general agreement with those authors who have found nerve cells in the interatrial septum and near the atrioventricular groove at the back and front of the heart, but not in the node or bundle of the dog, and presumably of man (DeWitt, '09; Woolard, '26; Blair and Davies, '35; Nonidez, '43). The possibility of small groups of nerve fibers following the bundle has not been precluded by this study, and these have been observed by some workers (Woolard, '26; Blair and Davies, '35; Nonidez, '43; Truex and Copenhagen, '47), but the main portion of the observable nerves to the ventricle seem to follow the coronary vessels. If nerves were conductile, the arrangement of nerve trunks is such as might be expected to produce base to apex excitation of the lateral walls of the ventricles, a situation which has been demonstrated to produce an abnormal type of electrocardiogram (Robb and Robb, '37; Stein, '45). Studies of the induction of reversed conduction (Kent, 1893), ablation studies such as those of Hering ('05), and embryological observations (His, 1893; Patten and Kramer, '33; Armstrong, '35) all indicate, to various degrees that other than nervous mechanisms are involved in cardiac conduction.

The path occupied by the specialized tissue in the dog, then, seems to represent the anatomical pattern which correlates best with the most readily demonstrable physiological phenomena of the heart. The distribution of this tissue in

the dog is similar to that described by various authors for other species, including man (Walls, '45).

Purkinje, in 1845, described the cells which bear his name. Since that time controversy has raged as to whether or not these cells are found in man and dog. Most of the subsequent observations did not use comparable techniques, and many of the distinctions made are consequently artificial (cf. Truex and Copenhaver, '47). Of greater importance than taking sides in this controversy is the identification of a tissue which consistently differs histologically from heart muscle and connective tissue, and which may be shown to possess continuity with other specialized tissue in a reasonably consistent pattern. This latter has been demonstrated in this study and reconstruction of the atrioventricular system of the dog.

Coincident with current studies on the electrical phenomena of the heart, chemical analyses of the various cardiac tissues, and comparative studies of the contractile mechanisms of cardiac and skeletal muscle, there is need for a complete comparative review of the anatomy of the heart in many species, accompanied by reconstructions so that a synthesis of facts may be possible in which observed structure and function may perfectly complement each other.

SUMMARY AND CONCLUSIONS

1. Detailed histological study of the upper septal portion of the atrioventricular system of a puppy has been carried out, and this tissue has been reconstructed in wax, as far as is known, for the first time. The general distribution coincides with that described by Tawara ('06) for various species.

2. The system has been observed in gross dissections of 7 fresh adult dog hearts to follow a course similar to that shown in the reconstruction. The diffuse origin of the left limb was borne out in each case.

3. Many direct transitions of specialized tissue to ordinary heart muscle were observed: e.g., the A.V. node to atrial

muscle; the right and central regions of the A.V. node, the main bundle, and the upper left branch to ventricular septal muscle; more peripheral transitions of the branches and sub-endocardial network to musculature of the walls.

4. Nerve cells and ganglia were seen in the upper interatrial septum and just anterior to the atrioventricular groove in the dorsal and ventral portions of the septum, but not in the A.V. node, the bundle, or its branches.

5. Small bundles of nerve fibers were observed in the interatrial septum in the region of the A.V. node, and their presence in the specialized tissue itself, while not demonstrated, is not precluded.

6. The major nerves to the ventricle seem to follow the coronary blood vessels.

7. At present, the specialized tissue of the heart seems to demonstrate best the anatomical relationships demanded of a conducting system for the mammalian hearts.

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PLATES

ABBREVIATIONS

AVB, Atrioventricular bundle	NA, Nodal artery
AVN, Atrioventricular node	PM, Papillary muscle
CFB, Central fibrous body	RB, Right branch of A.V. bundle
CS, Coronary sinus	RCA, Right coronary artery
LB, Left branch of A.V. bundle	RVB, Right branch to valve base
LVB, Left branch to valve base	ST, Specialized tissue
M, Cusp of mitral valve	V, Ventricular muscle
MS, Membranous septum	

PLATE 1

EXPLANATION OF FIGURES

- 1 Reconstruction of A.V. node, bundle, and main branches. $\times \frac{1}{4}$.
- 2 Detail of posterior surface of nodal area of reconstruction; Asterisk denotes region of end to end transition of specialized tissue to ordinary cardiac muscle. $\times \frac{3}{8}$ (approximately).
- 3 Photomicrograph of end to end transition of specialized tissue to ventricular muscle; transitional area shown by asterisk. $\times 242$.

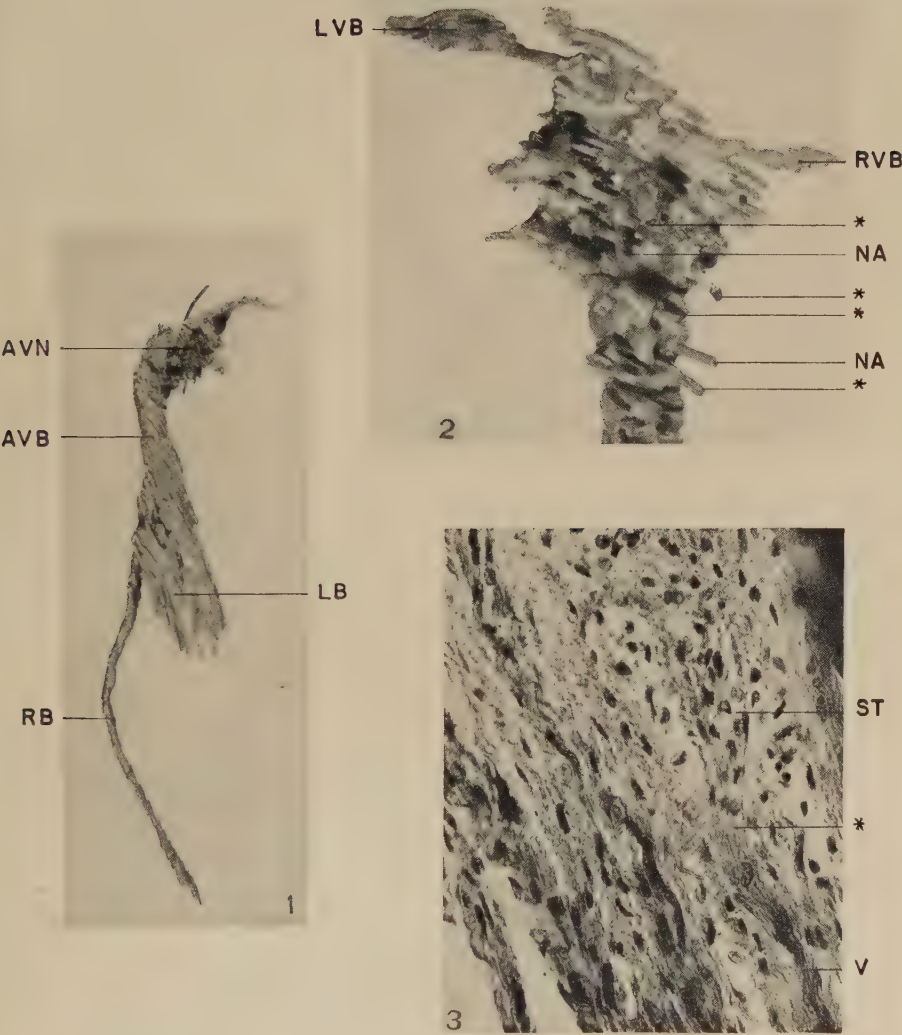
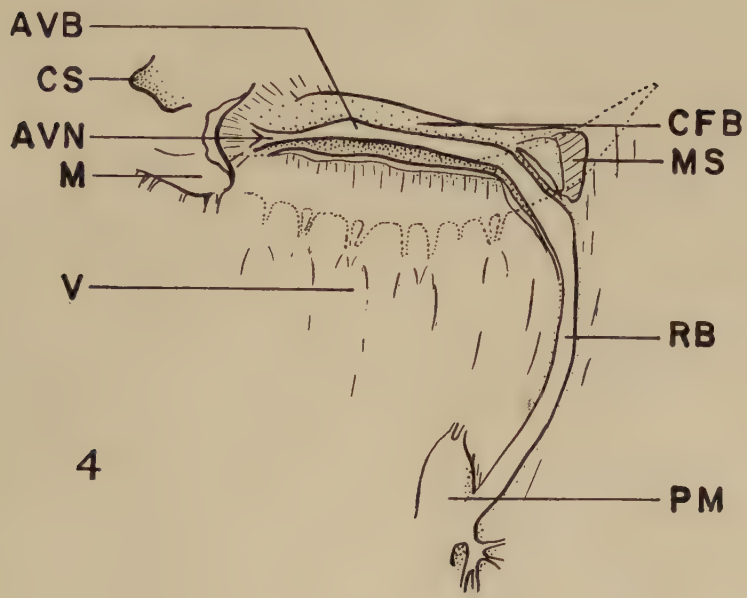


PLATE 2

EXPLANATION OF FIGURES

4 Drawing of gross dissection of A.V. system of dog no. 6, dissected from the right ventricle. Dotted line indicates position of medial cusp of tricuspid valve. $\times 1$.

5 Drawing of gross dissection of A.V. system of dog no. 7, dissected from the left ventricle. RB indicates point of origin of the right branch. $\times 1$.



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